THE INJURY OF TISSUE CELLS AND THE LIBERATION OF PHARMACOLOGICALLY ACTIVE SUBSTANCES BY THE TOXINS OF *CL. WELCHII* TYPES B AND C

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The toxin of *Cl welchii* type A liberates adenyl compounds from perfused organs but not histamine or other substances causing contraction of smooth muscle (Kellaway and Trethewie, 1941), whereas the toxin of *Cl. welchii* type D sets free both histamine and adenyl compounds (Kellaway, Trethewie and Turner, 1940). The first of these toxins is composed of α (Glenny *et al.*, 1933) and ζ (Prigge, 1936) fractions[2] and the second (under the conditions of cultivation used by us) of ϵ fraction. In the present paper we report a brief study of the toxins of the two remaining types of *Cl. welchii*, B and C, both of which usually contain α, β, γ and δ fractions, type B containing some ϵ fraction in addition.

METHODS.

The toxin of *Cl welchii* B was prepared by Dr. A. W. Turner of the division of Animal Health and Nutrition, C.S.I.R. It was made from a 20 hour culture in V.F. broth with 0·25 p.c. glucose of strain N.C.T.C. 3110 centrifuged and partly purified by precipitation with 65 p.c. ammonium sulphate and dried *in vacuo* over sulphuric acid. The m.l.d. for mice of 20 gm. was 0·04 mg. It was non-haemolytic and therefore probably contained no α fraction. The toxin of *Cl. welchii* type C was obtained from Dr. J. W. Trevan of the Wellcome Laboratories. It had a lethal dose for mice of 0·007 mg. The antitoxins used were prepared by Dr. A. W. Turner in goats. They neutralized 16,000 to 32,000 mouse m.l.d. per c.c. Since the antitoxin prepared from the goat against *Cl. welchii* type C caused severe circulatory effects in cats and contraction of the jejunum and uterus of the guinea-pig, particularly when mixed with toxin, we purified it by precipitating the globulin fraction. The globulins were precipitated by half saturation with ammonium sulphate, and the salt was removed by dialysis against distilled water for 48 hours. Toluol was used as a bacteriostatic. The purified globulin in amount equivalent to that in 0·5 c.c. of the original antitoxin had no action on the circulation of the cat nor any stimulant action upon the isolated jejunum and uterus of the guinea-pig.

Experiments on the circulation. These were carried out on cats and monkeys under chloralose anaesthesia with artificial ventilation and with the vagi divided. Changes in the pressure in the pulmonary artery were recorded by the method described by Kellaway and LeMessurier (1936). The venous pressure in cats was recorded from a cannula in a jugular vein and in monkeys in a femoral vein connected through a manometer containing saline with a Brodie's tambour. In these experiments chlorasol fast pink (2 c.c. of 8 p.c.) or heparin 3 to 5 mg. was used locally as an anticoagulant.

Perfusion experiments. The lungs of guinea-pigs were perfused with Tyrode solution as in the experiments of Feldberg and Kellaway (1937) and those of cats by the method described by Kellaway, Holden and Trethewie (1938), the hilum being dependent so as to allow of the collection of tracheal fluid. The perfusion rate was 1·5 to 2 c.c. per minute. The toxin was injected into the cannula in the pulmonary artery and kept in contact with the tissue for 4 minutes by interrupting the flow. The rate of flow was temporarily increased to between 4 and 10 c.c. per minute for the first 3 minutes after the flow had been re-established in order to dilute the excess of toxin in the perfusate. Samples of perfusate and tracheal fluid were boiled as soon as their volume had been measured. They were tested for histamine and slow-reacting-substance on the isolated jejunum of the guinea-pig suspended in oxygenated Tyrode solution in a bath of 7 c.c. and the titrations of histamine were checked by estimation on the carotid blood pressure of the atropinized eviscerated cat. The histamine estimated is expressed as di-chloride.

[1] This work was carried out under a grant from the National Health and Medical Research Council.

[2] Macfarlane *et al.* (1941) have recently pointed out that their work and that of Ipsen *et al.* has proved that the ζ toxin of Prigge is identical with the α toxin of Glenny *et al.* The non-haemolytic α of Prigge should now be called θ.

The liver of rabbits was perfused with Tyrode solution through the portal vein as described for the liver of the dog (Feldberg and Kellaway (1938)). Estimation of adenyl compounds in the perfusate was made on the heart of the atropinized guinea-pig by the method of Drury, Lutwak-Mann and Solandt (1938), the cardio-depressant effects being matched with adenosine (B.D.H.).

Before testing samples of perfusate for histamine and other muscle stimulant substances or for adenyl compounds we added antitoxin in amounts sufficient to neutralize toxins present in them. Extracts of organs for the estimation of histamine and adenyl compounds were made by grinding weighed amounts with finely divided silica in Tyrode solution (1: 50 or 1: 20) and were at once boiled.

Experiments on smooth muscle. These were made on pieces of isolated jejunum of the guinea-pig in oxygenated Tyrode solution and on single horns of the uteri of virgin guinea-pigs and rats. These horns were suspended in oxygenated anaphylactic Ringer's solution in a 7 c.c. bath.

RESULTS.

The Toxin of Cl. welchii *B.*

(1) *Circulatory effects.* In cats after the intravenous injection of 20 mg. of toxin there was a latent period of 5 to 10 minutes, then a steep terminal fall of systemic pressure accompanied by a sharp rise in the venous pressure. Immediately after the injection of 50 mg. of toxin there was a small and transient fall of

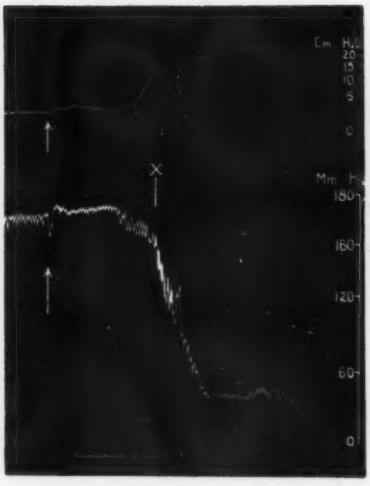

Fig. 1. Records of venous pressure (upper tracing) and carotid blood pressure (lower tracing) from a cat weighing 2·4 kg. At the arrow 50 mg. of the toxin of *Cl. welchii* type B were injected. X marks the onset of heart block. Time in ½ minutes.

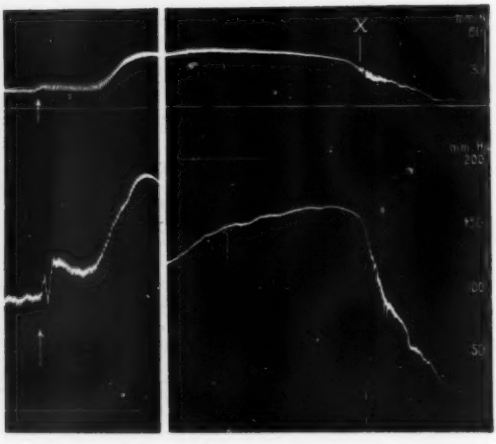

Fig. 2. Records of the pressure in a pulmonary artery (upper tracing) and in a carotid artery (lower tracing) from a cat weighing 2·6 kg. At the arrow 50 mg. of the toxin of *Cl. welchii* type B were injected. The interval between the right and left panels was 9 minutes. X marks the onset of heart block. Time in ½ minutes.

systemic blood pressure, and usually 2½ to 4 minutes later a steep terminal fall of systemic pressure, heart block being observed at its onset (Fig. 1). At the same time the pressure in the pulmonary artery and the venous pressure rose sharply. In two experiments a rise both in the systemic pressure and in the pressure in the pulmonary artery preceded the terminal fall of systemic pressure, the pressure in the pulmonary artery being maintained till death (Fig. 2). Apart from the trivial

immediate fall of systemic blood pressure attributable to a histamine-like impurity in the toxin all these circulatory changes were shown to be specific and did not occur when neutralized toxin was injected.

(2) *The Liberation of Histamine from Perfused Organs.*

(a) *The perfused lungs of the guinea-pig.* The injection of toxin of *Cl. welchii* B into the pulmonary artery of the perfused lungs of the guinea-pig does not cause the output of histamine or other muscle stimulant substance into the perfusate.

The lungs of a guinea-pig weighing 600 gm. were perfused with Tyrode solution and 50 mg. of the toxin of *Cl. welchii* B were injected. The sample collected during the first 3 minutes after injection contained too much toxin to allow of satisfactory testing but had no stimulant action on the isolated jejunum of the guinea-pig in a dose of 0·2 c.c. The samples collected during the next 3 hours were without action on the gut in doses of 1 c.c. as also was the drainage fluid (2·5 c.c.) collected when the preparation was cut down at the end of the perfusion. The weight of the lungs at the end of the experiment was 4 gm. which was reduced to 2·4 gm. by expression of the oedema fluid. A control piece of lung contained 50γ histamine per gm. and the lung at the end of the experiment contained 48γ per gm.

(b) *The perfused lungs of the cat.* The lungs of the cat are more sensitive to injury than those of the guinea-pig, and when they are set up the perfusate at first often contains histamine which diminishes in amount and finally disappears. The injections of toxin were not made until the perfusate had remained free from histamine for 30 to 40 minutes.

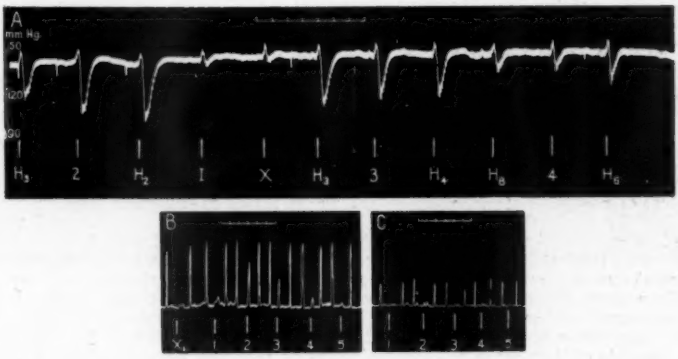

Fig. 3. Titration of perfusate from the lung of a cat after the injection of the toxin of *Cl welchii* B on the blood pressure of an atropinized eviscerated cat (A) and on the isolated jejunum of a guinea-pig (B). Titration on the isolated jejunum from another guinea-pig (C), of perfusates from a control lung in which neutralized toxin was injected. The unlettered responses in B are to 0·1γ and those in C to 0·05γ histamine. Time in minutes. Details in text.

The toxin of *Cl. welchii* B causes an immediate output of histamine into the perfusate lasting for from one to two hours. No other muscle stimulant substance is set free. The liberation of histamine is specific and does not occur if the toxin is neutralized before injection.

The lungs from a cat weighing 2·9 kg. were perfused until the perfusate was free from activity. During 40 minutes before the injection of toxin (50 mg. in 0·5 c.c.) neither histamine nor other muscle stimulant substance could be detected in it. The sample (12 c.c.) collected during the first 3 minutes after the injection of toxin contained 1: 2·5 millions histamine, that collected during the next 7 minutes (20·5 c.c.) contained 1: 4 millions histamine and that collected

during the next 20 minutes (46 c.c.) contained 1: 8 millions histamine. During the second half hour the sample (59 c.c.) contained 1: 25 millions histamine. During the next hour no appreciable amount of histamine was present in the perfusate. The drainage fluid (2 c.c.) contained 1: 10 millions histamine. There was no evidence of the output of any other muscle stimulant substance. The lung before the injection weighed 3·6 gm. and a control sample contained 40γ histamine per gm. of tissue. At the end of the experiment, after draining, the lung weighed 4 gm. and contained only 14γ histamine per gm.

The estimation of histamine in samples of perfusate from this experiment is illustrated in Fig. 3. The upper panel (A) shows the depressant effects on the blood pressure of the atropinized eviscerate cat of 1 c.c. amounts of a sample before the injection of toxin (1), and of the first, second and third samples after the injection (2, 3 and 4) together with the responses to 1 c.c. of histamine solutions 1 : 2 millions (H_2), 1 in 3 millions (H_3), 1 : 4 millions (H_4) 1 : 6 millions (H_6) and 1 : 8 millions (H_8). At X 4·2 mg. of neutralized toxin, the largest amount which could be present in 1 c.c. of the first sample after the injection was without effect. The lower panel (B) shows the responses of the isolated jejunum of the guinea-pig to 0·2 c.c. of a sample before the injection of toxin (1), and of the first, second, third and fourth samples after the injection (2, 3, 4 and 5). At X′ 1 mg. of neutralized toxin was without effect.

In control experiments the perfusate remained wholly inactive after the injection of 50 mg. of neutralized toxin. Fig. 3C shows the effect on the isolated jejunum of 0·2 c.c. of the samples from such an experiment before injection at 1 and after injection at 2, 3 and 4. The lung before the injection weighed 3·4 gm. and a control sample contained 25γ histamine per gram. The lung after the experiment weighed 4·1 gm. and contained 12γ histamine per gm. This low value was probably accounted for by loss during perfusion before the injection of toxin.

(3) *The liberation of adenyl compounds from the perfused liver of the rabbit.* The toxin of *Cl. welchii* B injected intraportally into the perfused liver causes the liberation of adenyl compounds together with an agent capable of inactivating them. It also causes a variable amount of depigmentation in the organ, liberating pigments into the perfusate.

A piece of rabbit's liver weighing 12 gm. was perfused with Tyrode solution, the perfusate being collected over a water bath at 95° C. Before the injection of toxin the perfusate was free from cardio-depressant substances. After the injection of 50 mg. of toxin in 0·5 c.c. the sample collected during the first 3 minutes (32 c.c.) contained no detectable amount of adenyl compounds; that collected during the next 7 minutes (13 c.c.) contained the equivalent of 2γ adenosine per c.c. During the next 20 minutes 42·5 c.c. were collected containing only traces of adenyl compounds. During the second half hour 37 c.c. were collected containing the equivalent of 4γ adenosine per c.c. During the next hour 82 c.c. were collected containing the equivalent of 2γ adenosine per c.c. A control sample of liver contained the equivalent of 1·14 mg. adenosine per gm. and the liver after perfusion contained 1 mg. adenosine per gm. Depigmentation occurred during the second half hour after the injection and pigment in small amounts was present thereafter in the perfusate. In other experiments in which the perfusate was collected at room temperature smaller amounts of adenyl compounds were detected in the perfusate after the injection of toxin.

In all these experiments more adenyl compounds were lost from the organ than could be accounted for by the total activity of the perfusate. The following experiment was designed to show the liberation by the toxin of an agent capable of inactivating adenyl compounds.

The perfusate from a piece of liver was collected at room temperature during 20 minutes before and during an hour after the injection of 50 mg. of toxin. To each of these samples adenosine was added to make a concentration of 1: 50,000. Portion of each boiled at once, served as controls and the remainder was incubated for 80 minutes at 40° C. Other portions were removed and at once boiled after 15, 30, 50 and 80 minutes. None of the samples containing perfusate collected before the injection of toxin showed any loss of activity as a result of incubation but those containing perfusate collected after the injection of toxin showed a gradual loss of adenosine which was barely appreciable after incubation for fifteen minutes. After thirty minutes incubation 20 p.c., after fifty minutes 30 p.c. and after eighty minutes 45 p.c. of the activity was lost.

In control experiments the intraportal injection of neutralized toxin into the isolated liver preparation failed to cause the liberation of pigments, of adenyl compounds or of inactivating enzyme.

(3) *Experiments on Smooth Muscle.*

(a) *The isolated jejunum of the guinea-pig.* The toxin of *Cl. welchii* B in doses of 2 to 5 mg. causes contraction of the gut which, when the Tyrode solution in the bath is changed, relaxes at about the same rate as after S.R.S. (slow-reacting substance, Feldberg, Holden and Kellaway, 1938). The latency for doses of this order is from 10 to 15 seconds. The excitability of the gut as measured by its response to histamine or S.R.S. is not diminished after repeated doses of toxin, but the responses to the later doses of toxin are diminished in extent. Fig. 4 I shows that though the toxin contains an impurity with some muscle stimulant action most of the effect is specific. At T, two doses of 5 mg. of toxin gave responses with latencies of 11 and 14 seconds, the second being diminished in extent. Between them at T′ 5 mg. of neutralized toxin caused a small response with a latency of 13 seconds. The antitoxin alone was without action upon the gut.

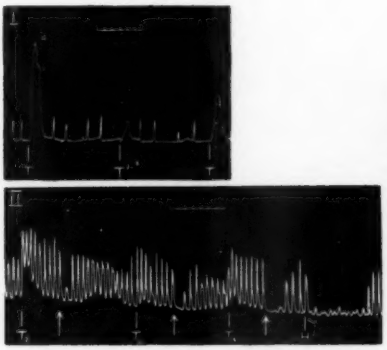

Fig. 4. I. Responses of isolated jejunum of guinea-pig to 5 mg. of *Cl. welchii* type B toxin at T and to 5 mg. of neutralized toxin at T′. The unlettered contractions are to 0·1γ histamine. Time in minutes.

II. Responses of one horn of the isolated uterus of a virgin rat to 20 mg. of type B at T_2 to 40 mg. of toxin at T_4 and to 20γ histamine at H. At the arrows the Ringer's solution in the bath was changed.

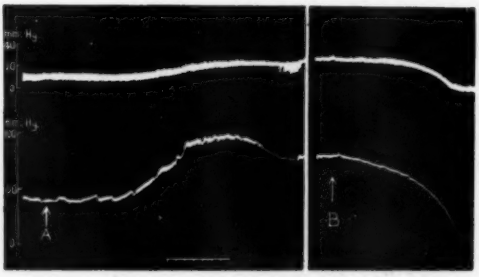

Fig. 5. Records of blood pressure from a pulmonary artery (upper tracing) and from a carotid artery (lower tracing) in a monkey weighing 2·25 kg. under chloralose anaesthesia with the vagi cut and artificial ventilation. At A 2 mg. and at B 8 mg. of the toxin of *Cl. welchii* type C were injected. The interval between the panels was 16 minutes. Time in ½ minutes.

(b) *The uterus of the virgin guinea-pig.* The toxin in doses of 10 to 20 mg. caused submaximal contractions of the uterus with latencies of 7 to 15 minutes. The preparation gave diminished responses to second and subsequent doses. These responses could not be shown to be specific, neutralized toxin causing responses of similar extent.

(c) *The isolated uterus of the virgin rat.* The toxin in doses of 20 to 50 mg. caused slight and transient contraction of the uterus followed by short-lived diminution of rhythm when the Ringer's solution was changed. When repeated doses

were given the contraction was diminished. The after-effect when the Ringer's solution was changed was shown not to be specific. Fig. 4 II shows the responses to two doses of 20 mg. at T_2 and to a third dose of 40 mg. at T_4.

The Toxin of Cl. welchii *C.*

(1) *Circulatory effects.* We have studied these effects in monkeys and cats on the systemic blood pressure, on the pressure in the pulmonary artery and on the venous pressure. They are generally similar in both species, moderate doses of toxin injected intravenously causing a rise followed by a steep final fall of systemic blood pressure, larger doses causing an immediate fall in blood pressure. The pressure in the pulmonary artery invariably rises and is maintained till just before death. The fall in systemic pressure whether it occurs early after large doses or late after moderate doses of toxin is accompanied by a rise in the venous pressure, and is sometimes preceded by a period of block.

Fig. 5 shows the effect of the intravenous injection of 2 mg. of *Cl. welchii* C at A in a monkey weighing 2·25 kg. There was a gradual rise in systemic pressure accompanied by a slow rise in the pressure in the pulmonary artery. A transient period of heart block is shown in the pulmonary arterial tracing at the end of the left-hand panel. This is not obvious in the systemic pressure tracing because of partial clotting. After an interval of 16 minutes during which the systemic pressure was maintained—a further dose of 8 mg. (3·5 mg. per kg.) at B caused death in 8 minutes. There was a progressive fall in the systemic blood pressure and a corresponding fall in the pressure in the pulmonary artery.

Very similar effects with doses of 2–4 mg. were observed in cats. These were shown to be specific, neutralized toxin being without effect on the circulation.

(2) *The Liberation of a Slow-Reacting Muscle Stimulant Substance from Perfused Organs.*

(a) *The perfused lungs of the guinea-pig.* The toxin of *Cl. welchii* C in doses of 15 to 30 mg. causes no detectable output of histamine but small amounts of a slow-reacting muscle stimulant substance somewhat similar to that formed by cobra venom (Feldberg, Holden and Kellaway, 1938) are set free in the perfusate.

The perfusate from the lungs of a guinea-pig weighing 580 gm. before the injection of toxin contained neither histamine nor any other muscle stimulant substance. Within three minutes of the injection of 30 mg. of toxin in 0·43 c.c. the lungs moved less freely but at the end of an hour after a good deal of fluid had flowed from the tracheal cannula they began to move better, collapsing during the expiratory phase. Samples of perfusate were collected during the first 3 minutes (10 c.c.), the next 7 minutes (9·5 c.c.) and the succeeding 20 minutes (29·5 c.c.) after the injection. During this last period 20 c.c. of tracheal fluid were collected. During the next half hour 40 c.c. of perfusate and 6 c.c. tracheal fluid, in the following hour 101 c.c. of perfusate and 13·5 c.c. of tracheal fluid, and in the final hour of perfusion 111 c.c. of perfusate and 4 c.c. of tracheal fluid were collected. The first two samples of perfusate after the injection contained too much toxin for satisfactory test on the guinea-pig jejunum. No histamine could be detected either in the perfusate or in the tracheal fluid but a slow reacting muscle stimulant substance appeared in the third sample of perfusate after the injection and persisted in the remaining samples. There was none in the first sample of tracheal fluid but it was present in the tracheal fluid collected with the fourth and fifth samples of perfusate. The drainage fluid collected when the lung was cut down (10 c.c.) contained 1: 7 millions histamine and a large amount of slow reacting muscle stimulant substance. The lung before injection weighed 3·7 gm. and after draining 6·4 gm. A control sample contained 18γ histamine per gm. and the content of the perfused lung after the injection was also 18γ, the oedema fluid having been removed by pressure between filter papers.

Fig. 6 shows the effect of some samples from this experiment upon the isolated jejunum of the guinea-pig. At 1, 1 c.c. of a sample of perfusate before the injection caused no response; neither did 0·25 mg. of neutralized toxin at X. At 3, 0·5 c.c. of the third sample after injection; at 4, 0·7 c.c. of the fourth sample, at 5 and 6, 1 c.c. of the fifth and sixth samples after injection were tested. At T3, 0·5

c.c. of the tracheal fluid collected during the third sample after injection, at T4, T5 and T6, 1 c.c. of the fourth, fifth and sixth samples of tracheal fluid were tested. The latencies of the resulting contractions were 5 to 7 seconds, and the after-effects on excitability of the gut slight. The output of muscle stimulant substance showed a gradual increase in the perfusate but after increasing in the tracheal fluid in the early stages decreased.

The liberation of this slow-reacting muscle stimulant substance by the toxin was specific, none being set free when neutralized toxin was injected. In these control experiments the ventilation of the lung was not impaired, there was no outflow of tracheal fluid, and only a slight degree of oedema of the lung.

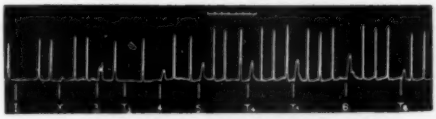

Fig. 6. Responses of the isolated jejunum of the guinea-pig to perfusate and tracheal fluid from the isolated lungs of a guinea-pig, after the injection 30 mg. of the toxin of *Cl. welchii* type C. The unlettered contractions are to 01γ histamine. Time in minutes. Details in text.

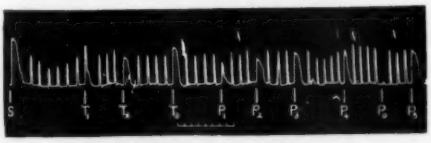

Fig. 7. Responses of the isolated jejunum of a guinea-pig to perfusate and tracheal fluid from the isolated lungs of a cat after the injection of 50 mg. of the toxin of *Cl welchii* type C. The unlettered contractions are to 0·1γ histamine. Time in minutes. Details in text.

(b) *The perfused lungs of the cat.* The injection of toxin of *Cl. welchii* C in doses of 15–50 mg. into the pulmonary artery of the perfused lungs of cats causes an output of histamine and of a slow-reacting muscle stimulant substance. Neutralized toxin gave rather smaller outputs of histamine, but no slow-reacting muscle stimulant substance could be demonstrated in the perfusate. The output of the slow-reacting muscle stimulant substance is therefore a specific effect of the toxin but the output of histamine is largely non-specific.

The presence of a muscle stimulating substance other than histamine in perfusate and tracheal fluid from a cat's lung weighing 9·8 gm. after the injection of 50 mg. of toxin is illustrated by the responses of the isolated jejunum of the guinea-pig in Fig. 7. At S is shown the response to 1 mg. S.R.S. left in contact with the gut for 30 seconds. This contraction had a latency of 7 seconds. The samples of perfusate and tracheal fluid (1 c.c.) were left in contact with the gut for 12 seconds. At T_1, T_2 and T_3 are shown the responses to tracheal fluid obtained between 15 and 35 minutes, between 2 and 2½ hours and between 2½ and 3 hours respectively after the injection. These responses were immediate owing to the presence of histamine but delay in relaxation after washing out must be attributed to the presence in increasing amounts of another muscle stimulating substance. The responses at P_1 and P_2 are to perfusate obtained between half and one hour and between one and one-and-a-half hours after the injection. Each had a latency of 5 seconds and is attributable to a muscle stimulant substance other than histamine. Samples of perfusate obtained between one-and-a half and two hours, between two and two-and-a-half hours and between three and three-and-a-half hours after the injection contained histamine in addition, the responses at P_3, P_4 and P_5 being immediate but relaxing slowly. At P_0 1 c.c. of perfusate before the injection was without effect upon the gut.

The liberation of histamine occurred regularly both when toxin alone and when toxin neutralized with antitoxin was injected. The output of histamine was always less after the injection of neutralized toxin and there was no outflow of tracheal fluid. For example in two experiments in lungs with similar content of histamine (42 and 40γ per gm.) in the first of which 50 mg. of toxin and in the second 50 mg. of neutralized toxin were injected the concentrations and amounts of histamine in the first three samples of perfusate collected 0–3 minutes, 3–10 minutes and 10–30 minutes after the injection were: after toxin, 1 in 4 millions (2·5γ), 1 in 4 millions (4·6γ) and 1 in 10 millions (4·4γ) and after neutralized toxin 1 in 10 millions (0·7γ), 1 in 9 millions (1·6γ) and 1 in 15 millions (3·4γ) histamine.

(3) *The liberation of adenyl compounds from the perfused liver of the rabbit.* The toxin of *Cl. welchii* C in doses ranging from 10 to 50 mg. injected intraportally into an isolated lobe of the rabbit's liver causes a loss of adenyl compounds from the organ (estimated by difference between control and perfused organ extracts). If the perfusate is collected at room temperature adenyl compounds are not detected in it, but if it is collected over a boiling water bath adenyl compounds can be estimated in the perfusate. Even with the smaller doses of toxin there is some depigmentation of the organ, pigment appearing in the perfusate 2 to 4 minutes after the injection. A thermo-labile agent capable of inactivating adenyl compounds is also set free into the perfusate. These effects of the toxin are specific, the injection of neutralized toxin failing to cause them.

A lobe of rabbit's liver weighing 20 gm. was perfused with Tyrode solution at a rate of 2 c.c. per minute and 50 mg. of toxin were injected intraportally. The perfusate was collected over a boiling water bath. Before the injection no adenyl compounds were detected in the perfusate. The sample collected during the first 3 minutes after the injection (33·8 c.c.) contained no detectable adenyl compounds; that collected during the next 7 minutes (11 c.c) contained the equivalent of 5·4γ adenosine per c.c.; that collected during the next 20 minutes (34 c.c.) contained the equivalent of 9·5γ adenosine per c.c. The perfusate collected during the second half hour (61 c.c.) contained the equivalent of 8·1γ adenosine per c.c. and during the third half hour (65·5 c.c.) 8·1γ per c.c. A control piece of liver contained the equivalent of 1·35 mg. adenosine per gm. The liver after perfusion and injection of toxin weighed 24·7 gm. and contained the equivalent of 0·87 mg. adenosine per gm. or correcting for oedema 1·08 mg. per gm.

In these experiments, as in those with cobra venom (Kellaway and Trethewie, 1940) and with other *welchii* toxins (Kellaway, Trethewie and Turner, 1940; Kellaway and Trethewie, 1941), the output of adenyl compounds in the perfusate was much less than the total loss estimated by difference between the control and perfused organ extracts. For example, in the above experiment the loss from the organ (estimated by difference of extracts) was 20 p.c. and the total amount estimated in the perfusate was 1·41 mg. or 5·2 p.c. of the total content of the organ. This is probably to be attributed, as in our experiments with cobra venom, to the action of inactivating enzyme in the vessels and tissues of the perfused organ.

A piece of rabbit's liver weighing 12·1 gm. was perfused with Tyrode solution for 20 minutes before and for 30 minutes after the injection of 50 mg. of the toxin of *Cl. welchii* C. The perfusate was collected at room temperature. To the samples before and after injection adenosine was added to make a concentration of 1: 50,000. Portion of each boiled at once served as controls. The mixtures were now incubated for 60 minutes at 40° C. and portions were removed and at once boiled after 15, 25, 40 and 60 minutes. None of the samples containing perfusate collected before the injection of toxin showed any loss of activity during incubation. In those containing perfusate collected after the injection of toxin after 15 minutes 30 p.c., after 25 minutes 45 p.c., after 40 minutes 55 p.c., and after 60 minutes incubation 70 p.c. of the activity was lost.

(4) *Experiments on Smooth Muscle.*

(a) *The isolated jejunum of the guinea-pig.* The isolated jejunum responds by contraction to this toxin in doses of 200γ or more. The contractions begin after latencies which decrease with increasing dosage, for example 90 seconds for 200γ, 15 to 20 seconds for 500γ and 10 seconds for 1 mg. Relaxation occurs slowly in 8 to 10 minutes. The excitability of the gut as measured by its response to histamine is depressed, and after large doses takes an hour or more to return to normal. After several small doses repeated at frequent intervals the gut may fail to respond to such a dose of toxin. In Fig. 8 II a piece of jejunum gave diminishing responses with a latency of 90 seconds to two doses of 200γ of toxin at C, and failed to respond to a third dose. This probably depends on the diminished excitability of the muscle, for after large doses given at intervals sufficient to allow recovery of excitability there was little diminution in the extent of the responses. Fig. 8 I shows that the contraction of the gut is a specific effect, 200γ of neutralized toxin at C′ causing only a trivial response.

(b) *The isolated uterus of the virgin guinea-pig.* The toxin of *Cl. welchii* C causes contraction of the uterus in doses from 200γ upwards. The latency of the response diminishes as the dose increases: for 200γ it is 90 seconds, for 400γ 30 seconds, and for doses between 1 and 5 mg. from 20 to 7 seconds. Relaxation is slow, requiring up to 20 minutes after doses less than 1 mg. and often more than an hour after doses of 5 mg. We were unable to desensitize the muscle to this toxin.

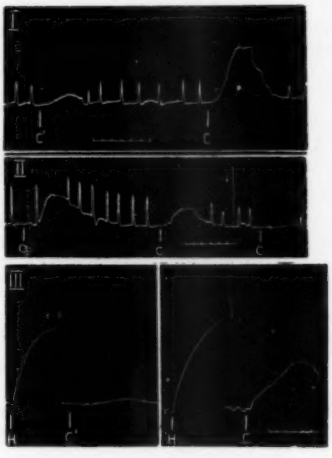

Fig. 8. Response of pieces of jejunum from two guinea-pigs (I and II) and of a horn of the uterus of a virgin guinea-pig (III). At C 200γ *Cl. welchii* toxin type C left in contact in I for one minute and in II for five minutes. At C′ 200γ neutralized toxin left in contact with the gut for five minutes. The unlettered responses are to 0·1γ histamine. In III at C 1·5 mg. of toxin and at C′ 1·5 mg. of neutralized toxin (left in contact with the uterus for five minutes) and at H 8γ of histamine added to the bath. Time in minutes.

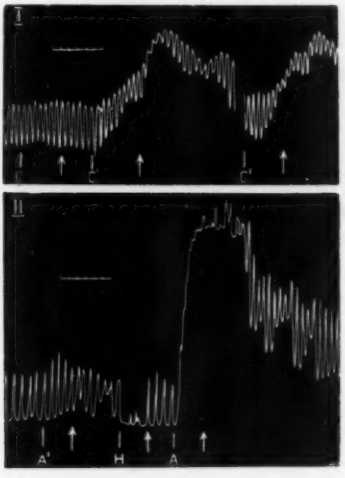

Fig. 9. Response of horns of the uteri of two virgin rats. In I at C 250γ of *Cl welchii* type C toxin at C′ 0·5 mg. of toxin added to bath. In II, 4 mg. of toxin at A and 4 mg. of neutralized toxin at A′ at H 20γ histamine. At the arrows the Ringer's solution was changed. Time in minutes.

Six successive doses of 5 mg. each left in contact with the uterus for three minutes were given at intervals varying from 35 to 60 minutes. The latency of the sixth response was 15 seconds, and though the latency was increased the extent of the later responses was not diminished. A dose of 10 mg. of toxin now gave a maximum response with a latency of 30 seconds. The contraction of the uterus is a specific effect. In Fig. 8 III 1·5 mg. of neutralized toxin gave an insignificant response, and after changing the Ringer's solution the same dose of toxin unneutralized caused a sub-maximal contraction with a latency of 20 seconds.

(c) *The isolated uterus of the virgin rat.* The toxin in doses from 0·5 mg. upwards causes strong contraction of the uterus attended by diminution or inhibition of rhythm. Relaxation (of the muscle) is slow. The uterus responds well

to repeated doses if adequate intervals are allowed for relaxation. Fig. 9 I shows the failure of the muscle to respond to 250γ toxin at C and the responses to two successive doses of 0·5 mg. of toxin at C′. The response to the toxin is specific. In Fig. 9 II 4 mg. of neutralized toxin at A′ had only a trivial effect, but at A the same dose of unneutralized toxin caused a large sustained contraction with initial inhibition of rhythm.

DISCUSSION.

It is not surprising that these two toxins exhibit a general similarity in their reactions since, apart from the presence of some ε fraction in B, they have a similar antigenic composition. Glenny and his colleagues (1933) regarded B toxin as being composed of the α fraction of A toxin, β, γ and δ fractions common to B and C and ε fraction of D toxin. They regarded C toxin as a complex of α, β, γ and δ fractions.

The toxins of each of the four types of *Cl. welchii* liberate adenyl compounds from the isolated perfused liver of the rabbit. The α or ζ and ε fractions must therefore possess this activity because A toxin is composed of α fraction with variable amounts of the ζ fraction of Prigge (1936), Nagler (1939) and the D toxin used in our experiments with Turner was almost wholly ε. Since the B toxin used in the present experiments did not contain the α constituent, its activity in liberating adenyl compounds must be attributable to the ε fraction present in it, to one or more of the fractions β, γ, δ or to some yet undescribed fraction.

The qualitative differences in behaviour between the toxins of *Cl. welchii* types B and C are that B causes liberation of histamine from perfused organs whereas C causes the liberation of a slow-reacting muscle stimulant substance other than histamine. ε fraction causes the liberation of histamine but α and ζ fractions do not, for D toxin so acts (Kellaway, Trethewie and Turner, 1940) and A toxin does not (Kellaway and Trethewie, 1941). It is probable that the liberation of histamine by B toxin is determined by the presence in it of ε fraction. The liberation of a muscle stimulant substance (other than histamine) which resembles but is not necessarily identical with the slow-reacting substance formed in tissues by snake venom cannot with certainty be attributed to any single fraction. Since it is liberated by neither A nor B toxin, its liberation cannot be attributed to α and ζ, nor to β, γ or δ fractions, unless one or more of these fractions in C are much more active quantitatively (in accordance with the greater activity of C) in this toxin than in B toxin. The liberation of this muscle stimulant substance may possibly depend on some as yet undescribed fraction in C toxin.

The circulatory actions of these two toxins have not been fully explored. Nicholson (1934) showed that both caused contraction of the coronary vessels, and it is probable that this action of the toxins, which is common to all the members of the group, is the dominant factor in the cardiac failure (evidenced in our experiments by heart block, steep fall of systemic and rise of venous pressure) which is the terminal event when these toxins are injected intravenously in cats.

The actions of C toxin on three varieties of smooth muscle differ from those of B toxin chiefly in the much longer time necessary after the former for relaxation. This appears to be a qualitative difference and cannot be explained by the greater activity of the C toxin. It may be related to the liberation of a slow-reacting muscle stimulant substance or may be attributable to some as yet undescribed antigenic constituent of C toxin.

SUMMARY.

The toxins of *Cl. welchii* Types B and C injected intravenously have similar effects on the circulation of cats, both causing a rise in pressure in the pulmonary artery and a terminal steep fall in systemic pressure often accompanied by heart

block and invariably by a sharp rise of venous pressure. The first effect with smaller doses of type C toxin is a prolonged rise of systemic blood pressure.

The toxin of *Cl. welchii* type B causes the liberation of histamine from the perfused lung of the cat, but fails to do so from the perfused lung of the guinea-pig. The toxin of *Cl. welchii* type C causes the liberation of a slow reacting muscle stimulant substance from both preparations. Both toxins cause the liberation from the perfused liver of the rabbit of pigment, of adenyl compounds and of a heat labile agent which inactivates these latter compounds.

Both toxins cause contraction of the isolated jejunum of the guinea-pig and of the uteri of the virgin guinea-pig and rat. These responses were shown to be specific except that of the isolated uterus of the guinea-pig to type B toxin—in which a histamine-like impurity was present.

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THE COBALT COMPOUNDS OF PROTOPORPHYRIN

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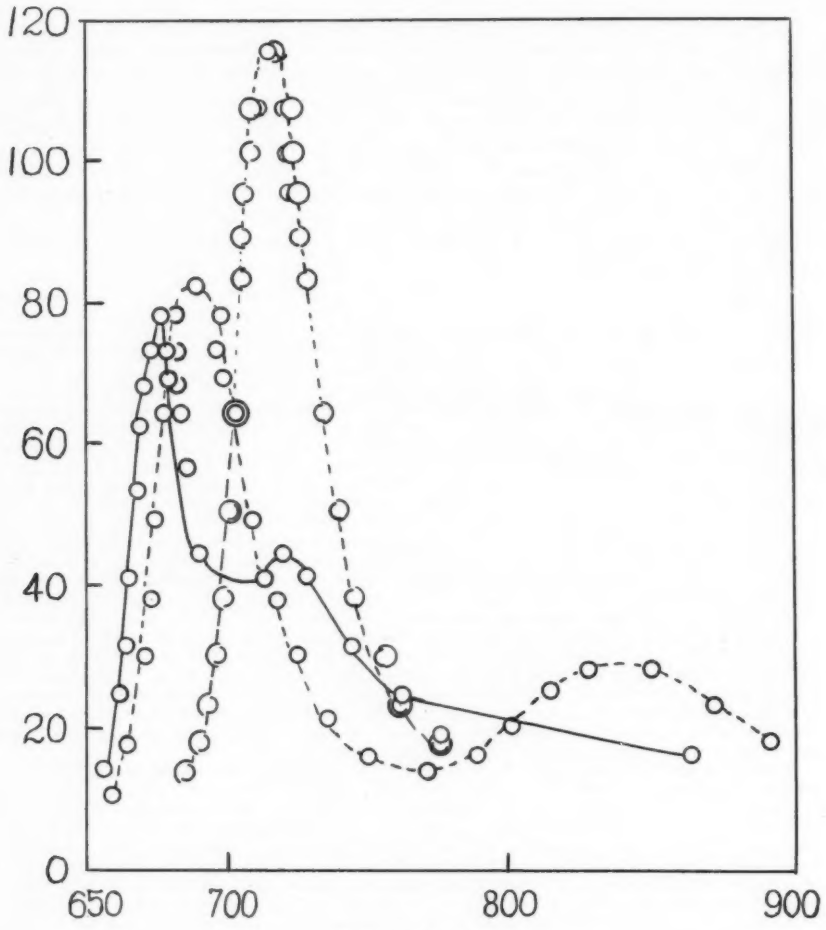
This work was done to observe the difference in derivatives of the blood pigment when the iron is replaced by so closely related an element as cobalt. Determinations were made with the Hartridge spectrometer of the positions of the visible absorption bands and with an ultra-violet spectrophotometer of the absorption in the nearer ultra-violet spectrum. For equipment and methods see Holden (1941). In spite of expressions of a contrary view from many sources cobalti-protoporphyrin derivatives can be reduced by $Na_2S_2O_4$ under conditions which do not denature globin. The reduction is slower than with haematin but nevertheless is sufficient for spectrometry if not perhaps for electrometry. Details typical of the more important solutions are given in an appendix, and the general results are now briefly discussed.

To explain many of the spectroscopic changes accompanying reactions of haematin it is assumed that haematin exists in solution in a colloidal state in dynamic equilibrium with a small amount of the pigment in individual molecules which react with globin and other nitrogenous substances, leading to a shift in the equilibrium until a new state dependent on the nitrogenous substance present is reached. An examination of the data for cobalt-protoporphyrin suggests that here the situation is different. The visible bands of cobalti-protoporphyrin globin are sharper and of shorter wavelength than those of free cobalti-protoporphyrin. Different specimens of both (see Table 1) show differences in wavelength according to the method of preparation and a study of the figures seems to show that most, if not all, of the specimens of the globin compound contain some admixed cobalti-protoporphyrin which is not in combination with the protein. This point is however further considered below. The free cobalti-protoporphyrin exists in varying states of aggregation according to the method of solution and the colloid aggregates are not in dynamic equilibrium with the active fraction. The cyanide compound of cobalti-protoporphyrin has like that of haematin a very different spectrum from its compound with such bases as pyridine. The latter has visible bands a little sharper and of shorter wavelength than those of the free cobalti-porphyrin and the ultra-violet band has a slightly higher extinction coefficient than that of the pigment dissolved with sodium hydroxide. When, however, cobalti-protoporphyrin is combined with cyanide the visible bands gradually change to a very faint α band and a broad and prominent β band. The ultra-violet band migrates to the visible region of the spectrum with an increase of about 170 A.U. in wavelength and can be seen by looking at a light rich in violet rays through a solution so dilute that the β band is barely visible. This effect is also shown by iminazole and 4 methyl-iminazole but not by 4, 5, iminazole dicarboxylic acid. It is not easy, in view of these facts, to consider that these substances combine with cobalti-protoporphyrin in quite the same way as bases or proteins which have relatively only a slight influence on the spectrum. The presence of an excess of pyridine inhibits combination with cyanide.

The following experiment shows other aspects of this situation. Into three rows of test-tubes were placed solutions of cobalti-protoporphyrin, 0·2 c.c. of 3 p.c. KCN, and 1 c.c. of 1 p.c. protein solution all adjusted to pH 9. The proteins were hen ovalbumin, casein, ox serum albumin and denatured ox globin. In the first row the cyanide was added half-an-hour after the porphyrin and protein solutions had been mixed; in the second the protein was added half-an-hour after the cyanide and porphyrin solutions had been mixed and in the third cobalti-protoporphyrin was added last. The tubes remained at 22° C. and were examined at intervals for three hours with a Hartridge spectrometer. At the end of a few minutes the three tubes containing ovalbumin showed the same spectrum, for casein and ox serum albumin over two hours was necessary while the tubes containing denatured ox globin were last. In this case the spectra indicated that little cyanide remained in combination with the porphyrin; with ovalbumin the spectrum was nearly that of free cobalti-protoporphyrin cyanide. With casein and with serum albumin the bands were of longer wavelength than the corresponding bands either of cobalti-protoporphyrin or of its cyanide, suggesting that compounds had been formed containing both cyanide and the protein. Other observations indicate that cobalti-protoporphyrin may combine with globin in two ways, one way shared with other proteins while the second appears to be specific for globin. If a solution of cobalti-protoporphyrin is mixed with one of globin there may be little change in the

[1] This work was aided by a grant from the National Health and Medical Research Council.

wavelengths of the visible bands, particularly if the porphyrin has been dissolved in dilute sodium hydroxide and the solution buffered to avoid denaturation of the globin. A freshly prepared solution, using the minimal quantity of dilute ammonia is the best for quantitative work but to prepare more concentrated solutions for qualitative purposes it is best to suspend crystals of cobalti-protoporphyrin in a solution of globin and to add a drop of 7N ammonia. The visible bands have, then, a wavelength of some 60 A.U. shorter than those of the free porphyrin. On none of these three solutions of cobalti-protoporphyrin globin has cyanide at a suitable reaction any effect, indicating that in each the porphyrin is linked to the protein. The fact that the last two



Ordinates $= \epsilon 10^{-3}$
Abscissae $= \nu 10^{-12}$

——o——o—— Cobalti-protoporphyrin and iminazole.

– – –o– – –o– – – Cobalti-protoporphyrin and cyanide. left curve

– – –o– – –o– – – Cobalti-protoporphyrin and globin. right curve
The large circles on this curve show points on the curve obtained by adding cyanide after the globin. The curves are virtually identical.

solutions differ from the first is shown by their behaviour towards reducing agents. When treated with $Na_2S_2O_4$ at pH 8·5 — 9 they are rapidly reduced to a compound with a band at 551 mμ while the first is slowly reduced to one with a band at 556 mμ the same as that of the free cobalto-protoporphyrin. On reoxidation of the first solution the bands of free cobalti-protoporphyrin reappear while the other solutions turn an olive green colour and a prominent band in the red is seen which is unaltered by subsequent treatment with $Na_2S_2O_4$, cf. Lemberg (1937). There appears no spectroscopic evidence for the combination of cobalto-protoporphyrin with cyanide, carbon monoxide, any nitrogenous base or any protein except globin. The difference between the ultra-violet spectra of cobalto-protoporphyrin and of its globin compound is small but in the visible region it is greater and the different behaviour on oxidation is confirmatory.

TABLE 1.

Wavelengths of bands in the visible spectrum.

		α	β	
A	Cobalti-protoporphyrin			
	NaOH	577	540	
	NH_3	575	538	
	Pyridine and NaOH	574	537	
	Globin	569	533	
	Cyanide	589	552	436
	Iminazole	575	538	443
	4 Methyl-iminazole	582	543	440
B	Cobalto-protoporphyrin			
	NH_3	566	—	
	Pyridine and NaOH	559	—	
	Globin	551	—	
C	Reoxidized cobalto-proto-porphyrin globin	636	572	536
D	Cobalti-protoporphyrin cyanide with proteins (see text)			
	Free	589	552	
	Ovalbumin	590	555	
	Ox serum albumin	597	560	
	Casein	594	559	
	Denatured ox globin	575	539	

TABLE 2.

The maxima of the ultra-violet bands of cobalti- and cobalto-protoporphyrin derivatives.

		$\epsilon 10^{-3}$	λA.U.
A	Cobalti-protoporphyrin		
	in NaOH	85	4,170
	NH_3	110	4,160
	pyridine and H_2O	97	4,190
	globin pH 8·5	115	4,200
	globin pH 5·2	107	4,190
	globin and KCN	115	4,190
	KCN and globin then Co-ppH	114	4,200
	KCN pH 6·5	58	4,335
	7·3	82	4,360
	8·0	82	4,360
	9·0	80	4,360
	9·6	80	4,370
	10·7	80	4,360
	KCN then globin after	83	4,210
	iminazole	78	4,430
	4 methyl-iminazole	66	4,400
B	Cobalto-protoporphyrin		
	in NH_3 pH 8·6	83	3,945
	globin pH 8·3	70	3,980
	cyanide	78	3,970

In calculating ε the accepted practice has been followed of regarding the crystalline cobalti-protoporphyrin as free with a molecular weight of 621 though since it is like haemin and unlike haematin insoluble directly in alcoholic hydrochloric acid the question is perhaps not finally settled.

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SUMMARY.

The spectra of cobalti- and cobalto-protoporphyrins and their compounds with globin, cyanide and iminazole are described.

APPENDIX.

Cobalti-protoporphyrin globin alone and with the addition of KCN:

stock = 1·05 mg. cobalti-protoporphyrin, 0·3 c.c. N.NH_3, H_2O to 10·0 c.c.
1·5 c.c. stock, 1 cc. rabbit globin solution (about 1 p.c.), H_2O to 5 c.c. pH 8·5.
1·5 c.c. stock, 1·0 c.c. rabbit globin solution, 0·2 c.c. M/15 KCN, 0·2 c.c. M/15 KH_2PO_4, H_2O to 5·0 c.c. pH 8·5.

Cobalti-protoporphyrin cyanide:

stock = 1·02 mg. cobalti-protoporphyrin, 0·4 c.c. 0·1 N.NaOH H_2O to 10 c.c.
0·5 c.c. M/15 KCN, 0·6 c.c. M/15 KH_2PO_4, 2 c.c. stock, H_2O to 5·0 c.c. pH 8·0.

Cobalti-protoporphyrin iminazole:

stock = 1·48 mg. cobalti-protoporphyrin, 0·4 c.c. N.NH_3 H_2O to 10·0 c.c.
1 c.c. stock, 0·1 c.c. N NH_3, 0·5 c.c. M/15 iminazole hydrochloride, H_2O to 5·0 c.c. pH 8·7.

THE INACTIVATING EFFECT OF SALICYLATE ON SUSPENSIONS OF SOME ANIMAL VIRUSES.

(HERPES, INFECTIOUS MYXOMA, SHOPE FIBROMA, VACCINIA, POLIOMYELITIS)

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INTRODUCTION.

Solutions of potassium salicylate inactivate certain plant viruses (Best, 1940). It was of interest, therefore, to test the effect of salicylate on animal viruses. If, as seems likely on the available evidence, animal and plant viruses do not differ greatly in their general nature, we might expect them to behave similarly towards salicylate. In any case similarities or differences in this respect would serve to compare the two types of virus.

Preliminary tests with Shope fibroma virus were sufficiently promising to warrant further work. The critical concentration of salicylate required to inactivate the virus in a given time was determined for four viruses, and the rate of inactivation was determined in two instances. A few tests were also carried out on the immunizing power of salicylate-treated virus. The results of this work are here reported.

EXPERIMENTAL METHODS.

Preliminary Tests for Uniformity of Test Animals, etc.

Skin variation. The variation in susceptibility of different areas of a rabbit's skin was tested by titrating Shope fibroma virus (OA strain) in four parallel areas from left to right across the back. No difference in the titre of the virus was observed.

Variation between different animals. The same virus-suspension titrated in different rabbits in ten-fold dilutions showed that a ten-fold difference between animals is not unusual.

Survival of virus at different temperatures. Portions of a 10 p.c. suspension of Shope fibroma virus were kept for 24 hours at temperatures of 4° C., 22° C. and 37° C. At the end of this period the titres were 10^{-4}, 10^{-3} and 10^{-5}, respectively. Subsequent tests with virus-salicylate systems were carried out at 22° C.

Preparation of virus-suspensions. Glycerinated tissue less than three weeks old was used in all cases. A 10 p.c. suspension in physiological saline was prepared and centrifuged at about 2,000 *g.* for 15 minutes.

Preparation of salicylate solutions. The stock solution was prepared by almost complete neutralization of the appropriate weight of A.R. quality salicylic acid with the required amount of a concentrated solution of KOH. The KOH was added slowly with vigorous stirring until the whole of the acid had dissolved and the pH value of the solution was 7·0 as measured with a glass electrode. The concentration of the stock solution was 2 M., and lower concentrations were prepared from it by simple dilution. The pH value of the lowest concentrations was about 0·3 unit lower than that of the most concentrated. No attempt was made to adjust this difference as it would be reduced by the addition of virus-suspension.

Method of setting up tests. Equal volumes of virus-suspension and of a solution of potassium salicylate of twice the final concentration desired were mixed rapidly in a test tube and left to stand at 22° C. for 24 hours. The suspensions were then inoculated into animals.

Plan for multiple titrations in rabbits. A preliminary experiment indicated that 0·5 M was an upper limit for the inactivation of Shope fibroma virus, and a further experiment was planned to determine whether there was a critical concentration for inactivation. A series of virus-

[1] Part of the expenses of this work was defrayed by the National Health and Medical Research Council.

salicylate mixtures was set up to cover the following range of salicylate concentrations: 0·05 M, 0·10 M, 0·20 M, 0·25 M, 0·30 M and 0·40 M. The final concentration of virus was 5×10^{-3}. Each mixture was titrated in ten-fold dilutions intradermally in rabbits. Virus controls with saline and two concentrations of potassium chloride were included to show whether the effects observed were due to the salicylate ion alone. The effect of salicylate at the site of inoculation was studied by adding to the virus, just before injection, salicylate to give a final concentration of 0·25 M.

EXPERIMENTAL RESULTS.

Multiple titrations in rabbits. Experiments based on the above plan were performed with Shope fibroma virus (OA strain), vaccinia and virus myxomatosum. The results are set out in Table 1.

TABLE 1.

Showing the inactivating effect on suspensions of various animal viruses of solutions of potassium salicylate, at pH 7 and 22° C.

Multiple intradermal titrations in rabbits.

(a) Time of contact = 24 hours.

Salt.	Molar conc. of salt.	Shope fibroma, OA strain. Test 1.	Shope fibroma, OA strain. Test 2.	Vaccinia. Test 1.	Vaccinia. Test 2.	Virus myxomatosum. Test 1.	Virus myxomatosum. Test 2.
K salicylate	1·00	0	—	—	—	—	
„	0·50	0	—	—	—	—	
„	0·40	—	0	—	0	?5×10^{-2}	?5×10^{-2}
„	0·30	—	0	—	0	?5×10^{-2}	?5×10^{-2}
„	0·25	—	0	5×10^{-3}	0	0	0
„	0·20	—	0	5×10^{-4}	0	0	5×10^{-2}
„	0·10	—	0	5×10^{-3}	5×10^{-3}	0	5×10^{-2}
„	0·05	—	5×10^{-4}	5×10^{-3}	5×10^{-3}	5×10^{-6}	5×10^{-7}
KCl	1·00	5×10^{-5}	5×10^{-5}	—	5×10^{-5}	5×10^{-6}	5×10^{-8}
„	0·25	—	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-6}	5×10^{-8}
NaCl	0·14	5×10^{-4}	5×10^{-5} / 5×10^{-5}	5×10^{-5}	5×10^{-5} / 5×10^{-5}	5×10^{-5} / 5×10^{-7}	5×10^{-6} / 5×10^{-6}

(b) Time of contact = 0 hours.

K salicylate	1·00	5×10^{-2}	—	—	—	—	—
„	0·25	—	5×10^{-5}	—	5×10^{-5}	5×10^{-6}	5×10^{-7}

0 = no reaction (virus inactive).
— = not tested.

In 24 hours *Shope fibroma virus* was completely inactivated by K salicylate at 0·1 M but not by KCl at 1·0 M. At 0·05 M salicylate it was only partially inactivated. Salicylate added immediately before inoculation had no effect on the titre at 0·25 M but with 1·0 M salicylate a considerable fall in titre was obtained.

In 24 hours *Vaccinia virus* was partially inactivated at 0·25 M K salicylate in the first test and completely inactivated at 0·2 M in the second test. Potassium chloride in 24 hours and salicylate added immediately before inoculation had no effect on the titre.

With *virus myxomatosum* the results were not clear-cut. In one test the virus was inactivated at 0·1 M salicylate, and in another at 0·25 M. Yet, in other rabbits, the 0·3 M inoculum gave a positive reaction with the highest concentration of virus (5×10^{-2}). In these tests virus was active only with the highest concentration of virus (5×10^{-2}) at salicylate concentrations of 0·1 M and 0·2 M. Generalization of lesions occurred about the sixth day, and reading became difficult. Necrosis at the site of inoculation was often caused by 0·3 M salicylate and invariably by 0·4 M and higher concentrations. The doubtful results in both tests at 0·3 M and 0·4 M salicylate may have resulted from localization of virus circulating in the blood in cells multiplying to repair damage caused by the higher concentrations of salicylate. Salicylate added immediately before inoculation caused some reduction in the titre in both tests.

Intradermal tests in individual rabbits. In view of the difficulties experienced in reading the lesions of virus myxomatosum when multiple titrations were made on a single rabbit, tests were made using a range of salicylate concentrations and inoculating each suspension (virus conc. 5×10^{-2} only) into individual rabbits.

The results are set out in Table 2. A wider range of salicylate concentrations was used but the several viruses studied were not necessarily tested at each concentration. The critical concentration for complete inactivation was not constant and the virus often remained active at higher concentration of salicylate than in the multiple titration experiments.

TABLE 2.

Illustrating the inactivating effect on suspensions of various animal viruses of solutions of K salicylate, at pH 7 and 22° C.

Time of contact = 24 hours.

Intradermal tests on individual rabbits.

Molar conc. of K salicylate.	Shope fibroma OA strain.			Vaccinia.			Virus myxomatosum.				
1·4	—	—	—	—	—	—	—	—	—	—	0
1·2	—	—	—	—	—	—	—	—	—	—	0
1·0	—	—	—	—	—	—	—	—	—	—	0
0·9	—	0	—	0	—	0	—	—	—	+†	—
0·8	—	—	0	0	0	—	—	—	—	0	0
0·7	—	0	—	0	—	0	—	—	—	0	—
0·6	—	—	0	0	0	—	—	—	—	+†	0
0·5	—	0	—	0	—	0	—	—	+*	0	—
0·4	+†	—	0	0	0	—	0	+	+	+	0
0·3	+*	?+	0	0	0	+*	+*	+*	+†	+	—
0·25	?+	—	—	—	—	—	+	+	—	—	—
0·2	?+*	+	+	0	0	+	+	+*	+	—	+
0·1	+	+	+	+	+	+	+	+	—	—	—
0·05	+	+	+	—	—	—	—	—	—	—	—
0·14 M NaCl control	+	+	+	+	+	0	+	+	+	+	+
	1	2	3			4	5	6		7	

1—0·2 M to 0·4 M lesions came up very late.
2—0·1 M to 0·2 M lesions came up late.
3—0·2 M lesion came up late.
4—control probably immune.
5—0·25 M and 0·3 M lesions smaller than control.
6—0·3 M and 0·4 M lesions smaller and appeared later than control.
7—0·4 M, 0·6 M and 0·8 M lesions later than control.

+ = virus active. 0 = virus inactive. * = "Satellite" lesion. † = lesion around area of necrosis. — = not tested.

Shope fibroma virus was inactivated at 0·3 M in two experiments but in a third it survived a concentration of 0·4 M.

Vaccinia virus was inactivated by 0·2 M salicylate in two experiments but not by a 0·3 M solution in another test, although in this case the lesion developed very late (10th day).

Virus myxomatosum was completely inactivated at 0·4 M in two experiments but in another was not completely inactivated at concentrations of 0·4 M, 0·5 M and 0·9 M. Lesions produced at concentrations above the region of critical inactivation indicated by the multiple titration experiments always came up later than the control lesion, and were usually smaller. It is likely that in the multiple titration experiments sufficient antibody was produced by the animal to inhibit the late manifestation of the small amounts of virus which remained active at these higher concentrations. As tissue suspensions were used, some virus particles may have escaped contact with salicylate and so remained active to initiate infection.

"Satellite" lesions were frequent at higher concentrations of salicylate. Two or three very small papules appeared late, and were situated at some distance from the site of inoculation.

The results with virus myxomatosum were especially irregular. However, the results of the multiple titrations and the tests in individual rabbits suggest that this virus is no less susceptible to salicylate than are those of Shope fibroma and vaccinia, but when single virus particles escape inactivation the susceptibility of the rabbit's tissue to virus myxomatosum is so great that infection readily follows. The fall in titre from 10^{-3} to 10^{-2} brought about by 0·10 M salicylate is equivalent to a 99·999 p.c. inactivation of virus.

Possible attenuation of virus. One rabbit received a myxoma virus suspension in 0·3 M salicylate but although it developed the disease it recovered. Three more passed through typical infections but recovered after doses as large as 5 ml. of this virus treated with 0·4 M salicylate. The mortality with the strain of virus used is normally 100 p.c. with even minimal doses.

Inactivation of herpes virus (HF strain) by salicylate. Preliminary tests showed that mice would not withstand concentrations of salicylate above 0·15 M, intracerebrally. Suspensions were set up to give final salicylate concentrations of 0·05 M, 0·1 M and 0·15 M. The effects of salicylate at the site of inoculation and of NaCl and KCl were determined as controls. All suspensions were titrated in ten-fold dilutions from 5×10^{-2} to 5×10^{-5} intracerebrally in mice (3 mice to each dilution). In test 1, glycerinated testicular virus was used and in tests 2 and 3, glycerinated brain virus was used. The results are set out in Table 3.

TABLE 3.

Showing the inactivating effect, on suspensions of Herpes virus, of solutions of K salicylate at pH 7 and 22° C.

Intracerebral titration in mice. Herpes virus. (3 mice to each dilution).

(a) Time of contact = 24 hours.

Salt.	Molar conc. of salt.	Testicular virus.	Brain virus.	Brain virus.
K salicylate	0·15	0	0*	0
"	0·10	5×10^{-4}	5×10^{-3}	5×10^{-2}
"	0·05	5×10^{-4}	5×10^{-4}	5×10^{-4}
KCl	0·10	5×10^{-2}	5×10^{-3}	5×10^{-4}
NaCl	0·14	5×10^{-2}	5×10^{-4}	5×10^{-4}

(b) Time of contact = 0 hours.

K salicylate	0·15	0	5×10^{-3}	5×10^{-3}

* Survivors tested for immunity by intracerebral injection of 1 p.c. virus. None immune.

The tests with mice showed that herpes virus was inactivated by concentrations of 0·15 M K salicylate. With rabbits as experimental animals the critical concentration appeared to be higher. To determine whether mice were less sensitive to the virus than rabbits the virus was titrated in mice and rabbits simultaneously. The same titre, 5×10^{-6}, was obtained in both. Later experiments in which most of the salicylate was removed by dialysis before inoculation gave the same critical inactivation concentration for the virus irrespective of whether the test was made in rabbits or mice.

Poliomyelitis. Virus-salicylate mixtures over the following range of salicylate concentrations, 0·1 M, 0·2 M, 0·4 M, 0·6 M, 1·0 M and 1·2 M were kept at 22° C. for 24 hours, dialysed at room temperature for approximately 18 hours and then inoculated intracerebrally in monkeys. The monkey which received the virus mixture with the lowest salicylate concentration, i.e. 0·01 M, developed no symptoms, while the remaining 5 monkeys all showed pyrexia, weakness and/or paralysis leading in one case to death. Because of this unexpected result the experiment was repeated, with similar results. However, subsequent tests for immunity by intracerebral inoculation of 1 ml. of a 1 p.c. suspension of poliomyelitis virus showed that of the monkeys which remained well, one was immune and one was non-immune. Monkeys which had shown only weakness without definite paralysis were all immune when tested. Controls developed typical symptoms after roughly the same period of incubation.

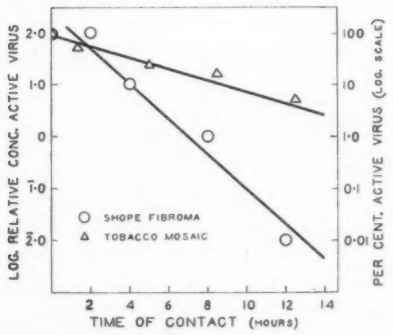

Fig. 1. Illustrating the rate of inactivation of Shope fibroma virus (OA strain) and tobacco mosaic virus by potassium salicylate.

A. Pure tobacco mosaic virus with 0·54 M K salicylate at pH 7 and 30° C.

B. Shope fibroma virus with 0·10 M K salicylate at pH 7 and 22° C.

The Rate of the Inactivation Process.

Shope fibroma virus. The loss of virulence of Shope fibroma virus in contact with 0·075 M and 0·1 M salicylate solutions was followed over a period of 24 hours. The results are set out in Table 4 and are illustrated in Fig. 1.

The titre of the virus in contact with 0·075 M salicylate remained relatively high over the 24-hour period of the test but the lesions were very much smaller than in the control. The titre dropped rapidly in the presence of 0·10 M salicylate, until after 12 hours' contact the amount of virus remaining active was of the order of 0·01 p.c. of the original value. After 24 hours' contact the virus had been completely inactivated.

Another experiment was designed to test whether Shope fibroma virus was inactivated by concentrations of salicylate below the critical if left in contact for time periods longer than 24 hours. The results for 0·075 M, 0·05 M and 0·025 M salicylate after 24 and 48 hour contacts are recorded in Table 4.

TABLE 4.

Showing the rate of inactivation of Shope fibroma virus (OA strain) and vaccinia virus by solutions of K salicylate, at pH 7 and 22° C.

Time of contact (hours).	Shope fibroma virus (OA strain). Molar concentration of K salicylate.					Vaccinia virus.	
	0·10	0·075	0·075	0·05	0·025	0·25	0·40
0	5×10^{-7}	5×10^{-7}	—	—	—	5×10^{-6}	5×10^{-6}
1	5×10^{-7}	5×10^{-7}	—	—	—	5×10^{-4}	
2	5×10^{-7}	5×10^{-6}	—	—	—	5×10^{-4}	
4	5×10^{-6}	5×10^{-7}	—	—	—	5×10^{-3}	
8	5×10^{-5}	5×10^{-7}*	—	—	—	5×10^{-3}	5×10^{-2}
12	5×10^{-2}	5×10^{-6}*	—	—	—	5×10^{-2}	
24	0	5×10^{-5}*	5×10^{-4}	5×10^{-5}	5×10^{-5}	5×10^{-3}	
48	—	—	5×10^{-5}	5×10^{-4}	5×10^{-5}		
	Controls for above tests. 0·14 M sodium chloride (physiological saline).						
24	5×10^{-6}	5×10^{-6}	5×10^{-4}	5×10^{-4}	5×10^{-4}	5×10^{-7}	5×10^{-6}
	0·075 M KCl.						
48	—	—	5×10^{-6}	5×10^{-6}	5×10^{-6}	—	—

* Reaction much less severe at these time intervals.

Vaccinia virus. The activity of a suspension of vaccinia virus dropped to 0·1 p.c. of its original value after 4 hours' contact with 0·25 M salicylate but did not appear to change further during the next 20 hours. In contact with 0·4 M salicylate the activity of the virus fell to 0·1 p.c. of its initial value after one hour's contact, but it did not appear to undergo any further change during the next 23 hours.

Effect of removing salicylate from inocula by dialysis. The lack of agreement between the results of different experiments in respect to the magnitude of the critical concentration of salicylate for *complete* inactivation may have been due to the presence of varying amounts of salicylate in the inocula at the site of inoculation. To test this possibility the greater part of the salicylate was removed by dialysis in a series of tests with the four viruses listed in Table 5.

TABLE 5.

Infectivity tests of virus—salicylate mixtures after reducing the salicylate concentration by dialysis.

Tests held at 22° C. for 24 hours, then dialysed for 7 hours at room temperature.

Molar concentration of salicylate before dialysis.	Intradermal tests on individual rabbits. Shope fibroma (OA strain).	Vaccinia.	Myxoma.	Intracerebral tests. Herpes (mice).	Herpes (mice).
1·40	—		0	—	—
1·20	—		0	—	—
1·00	0	0	0	0	0
0·90	—	—	—	—	—
0·80	0	0	+	0	0
0·70	—	—	—	—	—
0·60	+	+[1]	+	0	0
0·50	—	—	—	—	—
0·40	0	0	+	0	0
0·30	—	0	—	—	—
0·20	+*	0	+	0	0
0·15	—	—	—	0	0
0·10	+*	+	—	+	+
0·05	+	—	—	—	—
Saline control (0·14 M NaCl)	+	+	+	+	+

[1] = very small papule on 8th day.

0·2 and 0·6 animals immune. 0·3 and 0·4 non immune.

+ = virus active. 0 = virus inactive. * = "Satellite" lesion. — = not tested.

After setting up the virus-salicylate mixtures they were incubated at 22° C. for 24 hours and were then dialysed in cellophane cups against running tap water at room temperature for seven hours. The virus-saline control was not dialysed but was stored at room temperature during dialysis of the test suspensions. The increase in volume which took place during dialysis was greatest in the highest concentration. All suspensions were therefore adjusted with saline to the same volume. The suspensions were tested in individual animals.

It may be concluded from these results that differences in the amounts of salicylate present at the site of inoculation in experiments reported earlier in this paper were not responsible for the irregularities observed. The higher concentration required to inactivate completely Shope fibroma and vaccinia viruses, however, suggests that salicylate may have an inhibiting effect at the site of inoculation. It was observed that salicylate had an inhibiting effect on the diffusion (Duran-Reynals) factor with testicular virus suspensions and this may partly account for the effect of the salicylate at the site of inoculation.

Attempts to Produce Immunity with Salicylate-inactivated Virus.

Infectious myxoma. Attempts to produce immunity with myxoma virus inactivated by salicylate were unsuccessful.

Unglycerinated testicular virus was used in these tests as Cox and Olitsky (1936) obtained better results with fresh unglycerinated virus when immunizing guinea-pigs with formalized equine encephalomyelitis virus. Virus-salicylate mixtures of 1·0 M and 1·4 M salicylate concentration were kept at 22° C. for 24 hours and then dialysed at room temperature for approximately 18 hours. Five ml. of the virus-salicylate mixture were inoculated intramuscularly into rabbits. It was proposed to inject a similar dose at weekly intervals for 3 to 4 weeks and to test for immunity by intradermal inoculation 10 days after the last dose. Unfortunately all rabbits developed typical myxoma during the course of the inoculations; animals in a given batch did not, however, all develop myxoma after the same dose. This observation suggests (a) that the amount of virus capable of initiating infection in 5 ml. inoculum was very small and (b) that in animals developing myxoma after the 2nd–4th doses no effective immunity had followed earlier injections.

In the hope of completely inactivating the virus the above experiment was repeated keeping the virus-salicylate mixture for 8 days at 22° C. before dialysis and inoculation. All rabbits inoculated in this test developed myxoma after the second or subsequent doses. Titration of the mixture on a control rabbit did not cause myxoma and it seems that relatively few particles escape inactivation but these are sufficient to produce infection when large doses are given.

DISCUSSION.

The results show clearly that the viruses of herpes, myxoma, Shope fibroma and vaccinia are all inactivated by solutions of potassium salicylate; with 24 hours as the reference period, the greater portion had been rendered inactive by a tenth molar solution of salicylate.

From a chemical point of view the greatest interest attaches to the inactivation of the first 99 p.c. of virus. The method used to estimate relative virus concentrations is, however, only capable of distinguishing between ten-fold differences. About 90 p.c. of the virus must be inactivated before a diminution in activity is recorded, and so it comes about that most of the points on the rate of inactivation curve (Fig. 1) fall below the level represented by a 90 p.c. inactivation. Notwithstanding this limitation, common to all animal experiments of this type, the curve for Shope fibroma virus shows the rate of inactivation of this virus to be logarithmic, as is that of tobacco mosaic virus by salicylate (Best, 1940).

Curve A of Fig. 1 represents portion of an experiment with tobacco mosaic virus which was carried on for 10 days. Although after 12½ hours' contact with 0·54 M salicylate the activity of the tobacco mosaic virus had been reduced to 5 p.c. of its original value, some virus still remained active (a fraction of 1 p.c.) after 10 days' contact.

In the two experiments (Table 4) carried out with Shope fibroma virus with two concentrations of salicylate the rate of inactivation was logarithmic. An important consequence of this is that complete inactivation is approached assymptotically along the time axis. *Complete* inactivation in the mathematical sense is therefore approached only after infinite time and is a practical impossibility. In the sense used in biological work complete inactivation is attained for all practical purposes when the concentration of infective agent has been reduced to a level where it is incapable of initiating a disease in the most susceptible animal into which it is introduced. This is an arbitrary standard from which, however, there is no escape. Nevertheless, the arbitrariness of the standard for "complete biological inactivation", depending as it does to a large extent on the variability of the

test animals, is probably the chief cause of the relatively diffuse zone for the critical concentration for the complete biological inactivation of three of the viruses under investigation. The smaller the minimal amount of virus required to infect, the more diffuse may we expect this zone to be. The clear-cut results obtained with herpes virus may very likely indicate a comparatively large minimal infective unit.

From what has been said it is clear that the concentration of salicylate which brings about the rapid inactivation of the greater part of the virus is the more significant. However, it is also important to be able to define a limiting concentration above which "complete" biological inactivation occurs. The limit may be defined very precisely for herpes virus at 0·15M, for Shope fibroma and vaccinia viruses as approximately 0·7M, and for virus myxomatosum as about 1·0M. It is very likely that these latter figures would be lower and more precise if the work were repeated with purer suspensions of the viruses in the place of the crude extracts used in this work.

The circumstance that apparently inactive suspensions of virus still contain small amounts of active virus raises the question as to how far immunities induced by the injection of such suspensions are caused by the presence in them of subclinical amounts of active virus.

SUMMARY.

The viruses of herpes, Shope fibroma, vaccinia, and infectious myxoma undergo rapid inactivation when brought into contact with solutions of potassium salicylate of pH 7 at 22° C. In all cases more than 99 p.c. of the virus was rapidly inactivated at salicylate concentrations between 0·10M and 0·20M.

Poliomyelitis virus was not tested for partial inactivation, but complete inactivation was not brought about by even higher concentrations of salicylate.

The rate of inactivation was followed for Shope fibroma virus and was found to be logarithmic. It is pointed out that in consequence of this, complete inactivation can only be attained after infinite time, and a distinction is drawn between complete mathematical and complete biological inactivation. Herpes virus was rendered non-infective by 24 hours' contact with 0·15M salicylate at 22° C. and pH 7 when dialysed before inoculation, irrespective of whether the infectivity tests were carried out in rabbits or in mice. On account of the small residuum of active virus the limiting concentrations of salicylate necessary to render the other three viruses non-infective in 24 hours are not so well defined and are much higher. These, the influence of dialysis on the "end point", and a comparison with tobacco mosaic virus are discussed in the light of data presented.

Salicylate-treated virus myxomatosum was not an effective immunizing agent.

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INFLUENZA VIRUS INFECTIONS OF THE CHICK EMBRYO BY THE AMNIOTIC ROUTE

3. CHANGES IN THE ACTIVITY OF INFLUENZA VIRUS ON CONTINUED AMNIOTIC PASSAGE

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One of the most interesting characteristics of influenza virus is its capacity to develop with passage an increasing virulence for the particular host organism to which it is transferred. With repeated ferret passage, strains producing relatively severe pneumonia may be obtained. As a rule freshly isolated strains fail to produce demonstrable lesions in mice, but within a few generations fatal pneumonic consolidation develops, and with standard long-passage strains like "W.S.", "P.R.8." and "Melbourne" fatal infections are produced with highly diluted virus. In this laboratory we have studied particularly the changes associated with passage on the chorioallantois, and have shown that with both "Melbourne" and "W.S." strains highly virulent for the chick embryo can be developed (1936, 1938). The lesions produced in the embryo are haemorrhagic and particularly affect the brain. With the development of high virulence for the chick, the "Melbourne" strain lost its power to produce fever in ferrets though still capable of inducing immunizing infections. The egg passage strain of "W.S." however retained pathogenicity for both ferrets and mice.

In view of the likelihood that any effective method of immunization against influenza will require the use of suitably attenuated living virus given by the natural route, a study has been made of the effect of repeated amniotic passage in modifying the activity of two strains of virus for other experimental animals.

MATERIALS AND METHODS.

The two strains chiefly studied were those isolated from the Melbourne epidemic of July, 1939, "Bundoora" and "R". The characteristics of these strains have been described previously (Burnet and Lush, 1940). Both are of the antigenic type "Melbourne" but "R" is of wider antigenic range and has always shown itself of higher pathogenicity for the host organisms to which it has been adapted. The present experiments were started with first or second passage ferret-turbinates which had been stored in glycerol-Ringer solution for some months at a temperature a few degrees below zero.

The method of amniotic inoculation has been fully described previously (Burnet, 1940). During the early stages transfers were made at three or four day intervals but for the greater part of the series subinoculations were made at two day intervals. As a matter of convenience three passages per week have been done, the pooled allantoic and amniotic fluids being removed after two days incubation and transfer being made the same day or (once weekly) after being kept in the refrigerator for 24 hours. All fluids were cultured and all living embryos examined according to the usual technique, the macroscopic appearance of the lungs and the character of the cells in tracheal fluid smears being noted. Only on one occasion has it been necessary to revert to stored material on account of bacterial contamination.

Tests on mice, ferrets and by chorioallantoic inoculation have been made by the standard methods and antibody titrations of immune sera have usually been against "Melbourne" egg virus by the chorioallantoic technique.

Changes with influenza virus strain "R". The original filtrate from throat washings produced a typical ferret infection with a primary rise to 104·6° F. two days after inoculation and no secondary rise. Three ferrets receiving first or second passage material gave similar reactions, all reaching temperatures over 105° F. in the primary phase and only one showing a small second-

[1] This work has been carried out under grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

ary peak. Turbinates from most of these ferrets were stored in glycerol-Ringer solution in the freezing compartment of a refrigerator and the amniotic passage series was commenced approximately eight months later.

The reactions of first or second amniotic passage material in the form of pooled embryonic fluids taken usually three days after inoculation were as follows: The titre by amniotic titration was 10^7, i.e. at least 2×10^8 infective particles were present per c.c. of fluid. Ferrets gave a single spike temperature response with a somewhat variable incubation period, the temperature not reaching 105° F. and symptoms being mild (Fig. 1). Mice inoculated with undiluted material showed areas of consolidation but usually survived. We have previously described experiments (Burnet, 1941) to show that mice were immunized by the intranasal administration of fairly high dilutions of the unadapted virus. On the chorioallantois only a feebly granular appearance was produced, sufficient to indicate that specific infection had occurred though quite useless for quantitative work.

The first change noted on passage was the shortened survival time of the embryos. As with most strains subjected to passage, deaths within 48 hours began to appear about the seventh passage and from the twelfth onwards very few embryos given moderately large inocula survived beyond 60 hours. Most of these deaths were associated with a disappearance of nearly all the amniotic fluid. Once this stage was reached no further change in pathogenicity for the embryo was observed. It should be stressed that the development of lethal activity was quite unlike the development observed with long continued chorioallantoic passage of "Melbourne" or "W.S.". No haemorrhagic lesions in brain or muscles have ever been observed and apart from the lungs no macroscopic lesions were visible.

TABLE 1.

Mouse titration results with pooled fluids at various stages of amniotic passage.

Passage.	Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1st	—	2, 0, 0, 0	0, 0, 0				
16th		3+, 3, 3	2+, 1	1, 1, 1			
29th			43, 45, 46	3, 3, 3	3, 2, 1	2, 1, 0	1—, 0, 0
57th		46, 3	2+, 2+, 2	3, 2, 1	1, 1, 1	1, 1, 1	0, 0, 0
79th	3+, 3+, 3+	3, 3, 3	3, 2, 2	2, 2, 2	1, 1, 0		

The changes in virulence for mice are shown in Table 1. It will be seen that there was a progressive rise to about the thirtieth passage with subsequently a decrease in virulence. A notable feature of the mouse titration of the seventy-ninth passage is the wide range of concentrations producing non-fatal lung lesions. With an active mouse passage strain if the 10^{-3} dilution produced average grade 2 lesions all or nearly all of the 10^{-4} mice would be killed. The "low grade" activity of the virus was also evident from the appearance of the lesions particularly with dilutions 10^{-2} and 10^{-3}. The areas of consolidation were much smaller than usual with their edges less clearly defined giving the impression that relatively large amounts of a weak virus were concerned.

The virulence for ferrets at different stages is shown by the series of temperature charts depicted in Fig. 1. There are considerable irregularities but the general trend is clearly toward a diminution in the extent of the febrile reaction, two of the three ferrets inoculated with the final passages showing no significant rise of temperature. The antibody responses of these ferrets showed no consistent changes: against "Melbourne" egg virus titre was reduced under standard conditions to values ranging from 0·001 to 0·01 p.c. with two exceptions (F 129 twenty-third passage and F 149 seventy-seventh passage) which gave values of 0·04 and 0·035 p.c. One of the highest titres was given by a seventy-ninth passage ferret F 146 which showed no significant fever (0·0012 p.c.).

The changes on the chorioallantois showed a general similarity to those observed in mice. By the twentieth passage the strain was giving relatively good foci on the chorioallantois, titration of an amniotic fluid removed at two days showing for instance 12, 14, 17 foci at a dilution of 10^{-6}. The twenty-ninth passage material showed an average of seven foci at the same dilution and in general the number of foci has indicated the same approximate number of virus particles as would be deduced from the limiting dilution capable of infecting amniotically. The seventy-ninth passage showed little change but the foci appeared to be smaller and more irregular in size than those of the earlier passages. A direct comparison of fluids from second, forty-second and eighty-second amniotic passages of "R" on a single batch of embryos confirmed these changes. Relatively good foci were produced by the forty-second passage, the numbers indicating a titre of 5×10^7 infective particles per c.c. Faint granularity only was produced by the second passage although there were still some indications of activity with some of the membranes inoculated with the 1: 10^6 dilution. The eighty-second passage material showed poor foci and great irregularity in counts. As with the seventy-ninth passage titrations, a proportion of well developed foci appeared but these seemed to represent only a small proportion of the active particles.

Changes with strain "Bundoora". This strain was also described by Burnet and Lush (1940). Its amniotic passage series was initiated as in the case of strain "R" from first passage

ferret material stored for nine months in the refrigerator. The same rapid appearance of lethal infections resulted and in the later passages death of the embryo at 40 hours after inoculation was almost regular. Ferret virulence has always been of a lower grade than that of "R" and from the fiftieth passage no febrile responses were produced although the ferrets were substantially immunized as shown by their serum-antibody titres.

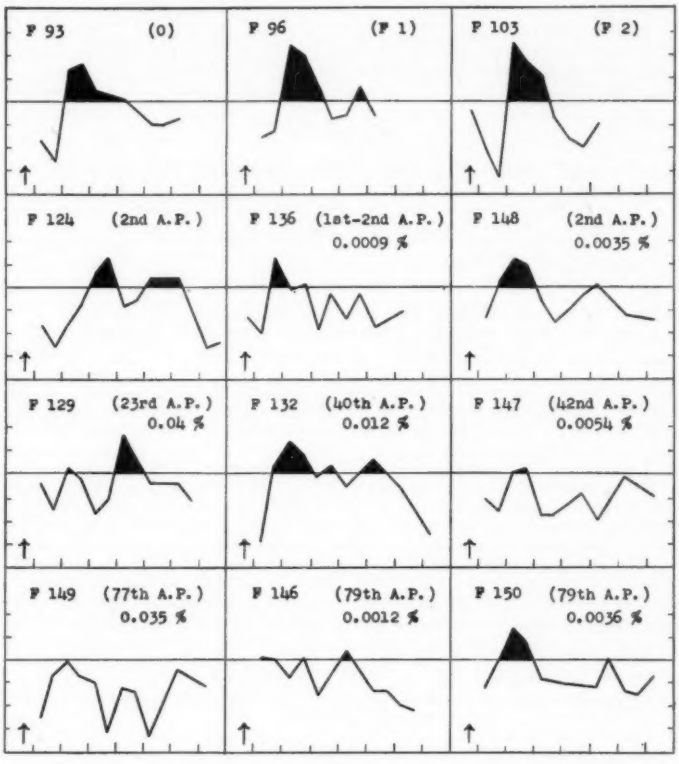

Fig. 1.

Change in ferret pathogenicity of strain "R" on amniotic passage.
Significant temperature rise over 103° F. shown in black.
0= original human material.
F 1, F 2 = first and second ferret passage.
2nd A.P. = second amniotic passage, etc.

Where estimated the titre of antibody against "Melbourne" egg virus is shown in the usual form of the percentage to which the activity of virus is reduced when mixed with undiluted serum.

No definite increase in mouse virulence has occurred. Undiluted virus of fortieth and fifty-first passages killed a proportion of the mice inoculated but the activity fell off rapidly on dilution and most mice inoculated with fluids diluted 1: 100 showed no lesions irrespective of the passage employed. At the eighty-second passage pooled amniotic fluids showed a power to infect embryos by the amniotic route at a dilution of 10^{-8}, the highest dilution tested. Mice given undiluted material showed rather ill-defined 2, 2, 1 grade lesions; with higher dilutions except for an occasional grade 1 lesion all the mice were unaffected. Ferret 151 given this material showed only one slight rise above 103° F. without symptoms. At no stage were more than vague granular lesions produced on the chorioallantois.

Changes in the ease with which inactivation by immune serum is affected. In a previous paper (Burnet, 1941) we showed that fully adapted amniotic passage strains showed little evidence of inactivation when immune serum-virus mixtures were tested by the amniotic route. With strains capable of producing mouse lesions mixtures "greatly over-neutralized" as judged by mouse test were still capable of infecting embryos by the amniotic route. The chief effect of the serum was to prolong the survival time of the embryos. With mouse strains of virus unadapted to amniotic passage the results of tests made in mice and by the amniotic method were essentially similar.

The only addition to the experimental facts that we have to record in this connection is concerned with unadapted human strains. A substrain of "R" re-isolated after experimental human infection was tested at its second amniotic passage against an homologous ferret immune serum. Table 2 shows that the serum effectively inactivates the virus.

TABLE 2.

Action of immune serum against a recently re-isolated human strain.

Virus alone.				Virus + immune serum F 143.			
10^{-1}	—	—	—	V 10^{-2} + S. und.	—	—	—
10^{-0}	(+)	(+)	—	V 10^{-2} + S. „	—	—	—
10^{-5}	+	+	+	V 10^{-1} + S. „	—	—	—
				V 10^{-1} + S. 1:5	(+)	—	—

\+ Typical lesions and positive tracheal smear.
(+) Death at third or fourth day—presumptive infection.
— No lesions after four or five days.

As far as we have gone it seems that those strains which kill a majority of the embryos inoculated in two or three days, are poorly neutralized by immune sera. Strains which require a longer period to produce death are inactivated when tested by the amniotic route almost as readily as in mice or on the chorioallantois.

Although the subject is not strictly relevant to the main topic of this paper we wish to report experiments which show that the virus inactivating agent of human nasal secretion (Burnet, Jackson and Lush, 1939; Francis, 1940) is capable of inactivating virus freshly isolated from human beings. All the previous experiments were done with well adapted mouse or egg strains and there was a possibility that fresh strains might possess a resistance to the agent. The same substrain of "R" re-isolated from a human infection was used and the test made according to the usual technique. Serial dilutions of virus were mixed (a) with an equal volume of serous nasal secretion sterilized by heating to 56° C. for 30 minutes and (b) with saline. The mixtures were held at 37° C. for five hours and then inoculated amniotically. Table 3 shows that typical inactivation occurs.

TABLE 3.

Inactivation of recently isolated human strain by V.I.A. (virus inactivating agent from human nasal secretion).

Virus dilution.	+ Saline.	+ V.I.A.
10^{-7}	(+) —	
10^{-6}	+ + —	
10^{-5}	+	
10^{-4}	+ + (+)	
10^{-3}		— — —
10^{-2}		— — —

Symbols as in Table 2.

DISCUSSION.

The chief aim of these experiments has been to develop strains of virus which might be suitable for human immunization with the particular problem in mind that might be presented by the appearance of a new antigenically distinct strain of virus. With rapid amniotic passage it might be possible to develop a sufficiently attenuated strain within the few weeks that would be available. The pathogenicity of strains at different stages of adaptation is at present being tested in human volunteers with low circulating antibody, but the present communication is concerned only with laboratory work.

The chief point of interest in our results is the rapid development of an acute lethal effect on the embryo. In our earlier work with chorioallantoic passage the lethal effect which eventually developed was associated with spread by the blood stream and acute damage to the vascular endothelium particularly in the brain, muscles and developing feathers. With amniotic passage no such haemorrhagic lesions have been observed. An occasional embryo shows some haemorrhage in the feather shafts but we have seen this also in poorly developed embryos without specific infection, and it is quite unlike the typical haemorrhages of a fully adapted chorioallantoic strain. The only suggestion we can make in regard to the cause of death after amniotic inoculation is that it may be due to the acute liberation of products of cell damage in the lungs. We have often observed that in mice given very large doses of active mouse virus death usually occurs in three days with only incomplete consolidation of the lungs. With smaller doses causing death in five or six days the lungs are almost invariably found to be completely consolidated. Since life can apparently be maintained with only a minimum of functional tissue in the lungs the early deaths must be due to some other factor. It seems reasonable to assume that with very acute infections the liberation of products of cell damage may be so rapid as to cause death either in mice or in chick embryos, the essential feature being the rapidity of the damage rather than its extent. On this view the changes observed with passage are mainly a result of the increased speed with which the virus can infect and multiply within the susceptible cells. The protection against early death afforded by immune serum can then be ascribed simply to a slowing-down of the processes of infection.

Changes in the virulence for other host organisms appear to vary from strain to strain. In general our experience has been that adaptation to one host results in a loss of virulence for others. This may at times be more than counterbalanced by the fact that adaptation may be associated with a big rise in the titre of the usual stock virus which is sufficient to mask any slight qualitative fall in virulence. In the case of strain "R" the development of activity in mice and on the chorioallantois was not solely a quantitative phenomenon; some qualitative change must have undoubtedly occurred. With "Bundoora" no such increase in heterologous virulence occurred, the variations in the mouse lesions produced being only such as might result from minor differences in titre of the preparations tested.

SUMMARY.

Changes in the activity of two strains of influenza during the course of amniotic passage are recorded.

A rapid increase in virulence for the embryo occurs but no haemorrhagic lesions are produced.

The power to produce symptoms in ferrets becomes progressively diminished, but immunizing power is essentially unaltered.

With one strain "R" virulence for mice rose to a peak around the thirtieth to fortieth passages and thereafter diminished.

A freshly isolated human strain tested by the amniotic method was readily inactivated by immune serum and by V.I.A.

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STUDIES IN RESPIRATION

4. THE EFFECT OF VARYING THE TIME OF APNOEA ON THE OCCURRENCE AND EXTENT OF THE OXYGEN TROUGH OF EXPIRATION

by F. S. COTTON

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(Accepted for publication 22nd February, 1941.)

Three subjects were selected who had previously exhibited this phenomenon to a marked degree, and whose maximum limits for apnoea varied considerably. Thus whilst the limit for J.M. was 40 seconds, W.S. could hold his breath for more than two minutes, and F.S.C. for one-and-a-half minutes.

METHOD.

The apparatus and detailed procedure have been described in previous communications (Cotton, 1939, 1940). The expired air was collected in the smaller of the two systems of tubes described, namely that consisting of tube units, each about 130 ml. capacity, connected by T pieces for sampling. On the graphs therefore, the sample obtained at position n represents the composition of expired air when approximately 130 ml. remained unexpired.

Experiments were conducted under standard conditions, varying only the time of apnoea. The apnoea times chosen for the experiments of each series depended upon the limit of the subject. (Table 1).

TABLE 1.

Times of apnoea in seconds.

Experiments not necessarily in same numerical and time order.

No. of Ex.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
J.M.	0	5	10	15	25	35	40	—	—	—	—	—	—	—	—
F.S.C.	0	0	10	10	20	20	30	30	40	40	50	60	70	80	90
W.S.	0	15	30	30	40	60	80	90	120	120	120	—	—	—	—

Since the results in the case of W.S. are the most striking they are shown in detail in Fig. 1, where each curve is plotted with a common abscissa for volume and with the same vertical scale for p.c. of oxygen. The absolute values of oxygen percentages are, however, different in the case of each curve for any given vertical height since the position of the initial value for each curve has been so chosen, as to show them all as a readily comparable system. To prevent overcrowding, the two 30″ experiments are averaged while the 15″ and the 80″ experiments are omitted.

Conclusions deduced from these data are: 1. The depth of the oxygen trough increases progressively with the time of apnoea. 2. The point at which the oxygen gradient is reversed, occurs earlier in the expiration as the apnoea period increases. 3. As the apnoea period increases the points on line 16 fall more extensively than the points on line 1. This becomes significant if we regard each of these separate experiments as representing progressive changes of lung air in a single 120″ apnoea experiment. It then implies a quicker drop in the oxygen percentage in that portion of lung air to be expired early than in the portion to be expired later.

Since the absolute values of the various curves cannot be compared in Fig. 1, Fig. 2 is constructed with the same ordinate and abscissa for each curve.

Excepting the experiment with 80″ apnoea each curve (representing a separate experiment) lies below its predecessor in apnoea time. The two 30″ experiments are again averaged since they lie close to each other throughout. One only of the three 120″ experiments was analysed for many samples. The curve shown is for this experiment but the oxygen trough values for all were used in plotting subsequent graphs.

The trend of the trough position is shown by the broken line.

The relation between time of apnoea and depth of the oxygen trough is shown in Fig. 3, curve A, which also includes the data from the earlier experiments after 120″ apnoea. In this case the best fit for the points is a straight line. Curve B is the corresponding graph for CO_2.

The sample which contains the minimum percentage of oxygen in a series showing the oxygen trough phenomenon may be called the trough sample and the sample in such a series which has the maximum O_2 percentage after the trough sample may be called the crest sample. In these terms, the extent of the oxygen trough is the difference in oxygen percentages of the crest and the trough samples, expressed in units of 0·01 p.c. Similarly the difference in CO_2 p.c. in the trough and crest samples may be called the CO_2 hump. Thus in curve B each value for CO_2 represents the CO_2 hump for that experiment in question.

The curve for the CO_2 hump is not drawn as a straight line since this would give negative values for apnoea times approaching two minutes. In none of the experiments carried out with W.S. however, did any negative values occur for the CO_2 hump, so that the final trend is drawn as a curve approaching but not cutting the zero line.

There is little doubt that the two curves show opposite trends.

As the time of apnoea increases the oxygen trough increases, with no apparent tapering off, so that its maximum value is determined by the apnoea limit of the subject. On the other hand, in the case of the CO_2 hump, in spite of irregularity of the constituent points, the general distribution justifies the drawing of a curve descending from left to right, so that the CO_2 hump tends to disappear as the apnoea limit is reached. In this case a large oxygen trough is associated with a small CO_2 hump while a small oxygen trough is associated with a relatively large CO_2 hump.

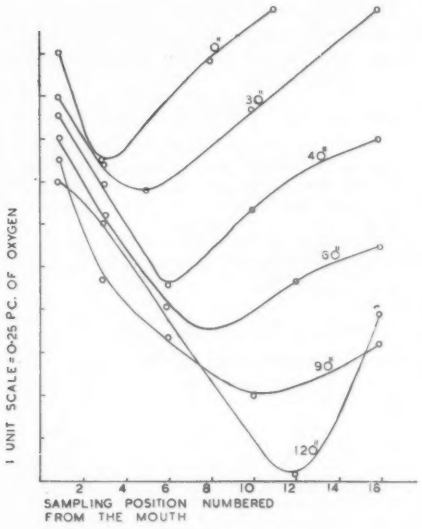

Fig. 1.

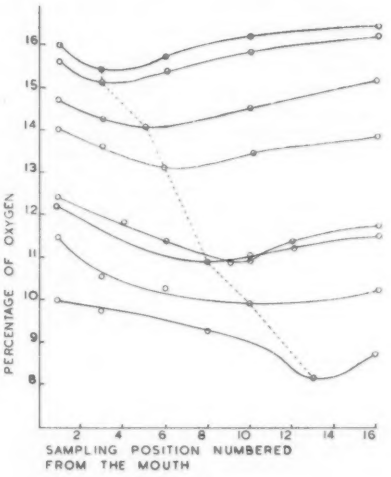

Fig. 2.

The relationship between time of apnoea and the position of the deepest point in the oxygen trough is set out in Fig. 4. Here there is good agreement between the points, indicating linear relationship. One implication of this curve deserves emphasis, namely, that in the longest apnoea experiment, when the trough point has been reached in expiration there remain about 1,600 ml. of the supplemental air having a greater percentage of oxygen than the air immediately expired. There still remains to be considered the residual air, which would probably be not less than about 1,300 ml. in this subject.

At present we have no way of determining the composition of this residual air. Unless however, the curve again changes direction very radically, we see by summing the supplemental and residual air, that at the trough point the whole contents of the lung, approximating 3 litres, may be richer in oxygen than that just expired.

In contrast to the findings above, in the case of the second subject of Table 1, many experiments have been carried out without producing any appreciable effect on the position of the oxygen trough in relation to the expired air. In this case we need only consider the effect of the apnoea period on the magnitudes of the oxygen trough and CO_2 hump. These are shown in Fig. 5. Here the values for experiments with the same apnoea period have been averaged in each case. These results are in accord with those of W.S. in that the oxygen trough increases progressively with the time of apnoea. There is, however, a point in contrast in that here the curve seems to flatten out at the top, so that it is doubtful whether, if apnoea time could have been increased, there would have been a further increase in the oxygen trough. On the other hand, the trend of the CO_2 hump runs in this case in the same direction as the oxygen trough, and in this respect the two subjects W.S. and F.S.C. manifest contradictory phenomena.

On account of the very irregular scatter of the CO_2 values in the graphs for each subject, the matter demands further examination. This irregularity does not necessarily imply irregularity within the individual experiments. An examination of the intra-experimental regularity shows

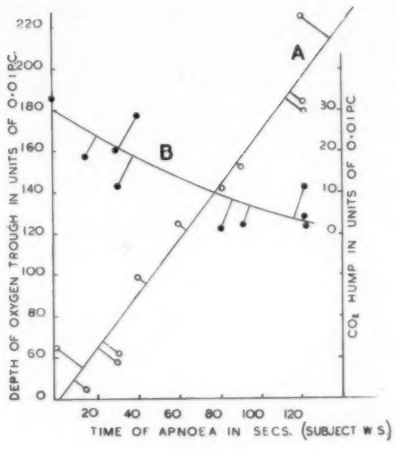

Fig. 3.

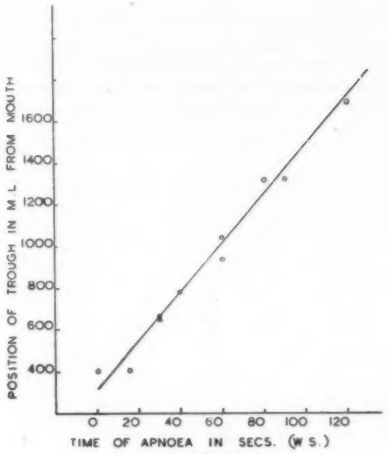

Fig. 4.

that the contrary is the case, for the CO_2 values in any given experiment, form a very smooth curve when plotted as usual in relation to the volume expired. This may be illustrated by taking two of the shorter and two of the longer apnoea experiments with W.S. The CO_2 curves in these four cases are shown in Fig. 6. These curves are all shown for comparison on the same vertical scale, with breaks in this ordinate to bring the series within a reasonable compass.

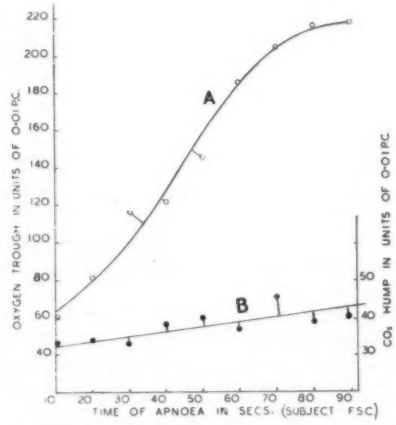

Fig. 5. A. Graph of oxygen trough. B. Graph of CO_2 hump.

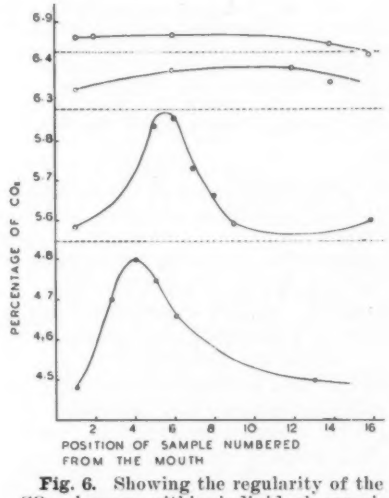

Fig. 6. Showing the regularity of the CO_2 changes within individual experiments. The two upper curves are for short apnoea periods. The two lower curves are for long apnoea periods.

It appears that the variation between experiments which leads to the irregularity shown in the B curves of Figs. 3 and 5, is due to some factor at present beyond our control.

A similar exploration of the sets of CO_2 curves for intra-individual experiments in the case of F.S.C. shows that smooth trends result here also, thus confirming the reliability of the CO_2 curves in Fig. 6.

This stabilization of the sets of CO_2 values for each subject confirms the conclusion that the trends for the CO_2 hump are real, so that an additional and entirely different aspect of the oxygen trough phenomenon would seem to be indicated here.

In the case of the third subject J.M. less data are available for analysis. The result, however, agrees with the previous findings in regard to the oxygen trough, namely that it is greatly influenced by the time of apnoea and increases progressively with the apnoea. Fig. 7.

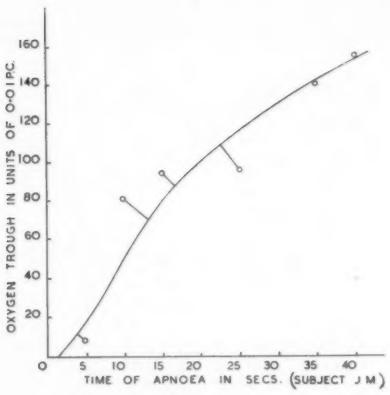

Fig. 7.

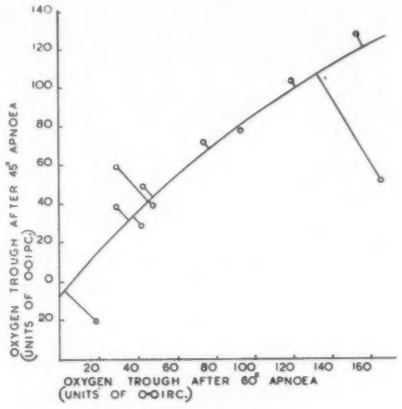

Fig. 8.

Another series of experiments was conducted to predict the oxygen trough of subjects after 45″ apnoea, from the oxygen trough already measured after 60″ apnoea. This was done to compare the results obtained with athletic and non-athletic subjects. In these experiments some were unable to complete the standard 60″ apnoea experiment, so that an apnoea period of 45″ had to be adopted for them. The result in Fig. 8 indicates that the mean oxygen trough after 60″ apnoea is from 10–20 p.c. greater than that after 45″ apnoea. Hence the results with an extended group supports the findings in the intensive investigations of the 3 subjects.

DISCUSSION.

The major part of the present work has been confined to three subjects since the location of a single point on a curve requires a complete experiment. None the less, the manner in which the oxygen trough increases with apnoea time, and the results from the group comparisons of a positive increase in trough from 45″ to 60″ apnoea, leave no room for doubt that the relationship stated is both real and general. Future experiments manifestly should be made uniform both with regard to apnoea period and duration of expiration.

Attempts to explain the individual differences between the results of W.S. and F.S.C. in regard to the opposite trends of the carbon dioxide hump, with increasing apnoea, and in regard to the respective migration and non-migration of the trough position must await a better understanding of the fundamental phenomenon.

SUMMARY.

It has been found in the case of three subjects that the depth of the oxygen trough of expiration increases progressively and profoundly with increase of apnoea time.

Confirmation of the generality of this change was obtained in a separate series of observations on eleven additional subjects, where it was shown that the oxygen trough after 60″ apnoea tends to be greater by 10 to 20 p.c. than that after 45″ apnoea.

The magnitude of the CO_2 hump may either increase or decrease in different subjects, with increase of the time of apnoea.

The location of the lowest point of the oxygen trough in terms of volume of air expired differed profoundly in the cases of the two main subjects of investigation.

With one subject the location remained practically constant in contrast with a second whose trough point migrated steadily with increasing apnoea away from the mouth end of the tube.

The data are insufficient to afford a basis of explanation for these phenomena.

Acknowledgments. Thanks are due to the subjects who so willingly co-operated, and especially W.S.[1], whose ability to endure repeated and prolonged apnoea without preliminary forced breathing, proved invaluable.

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[1] The unique respiratory experiment of W.S. is worthy of special record, since it may later be shown to be of significance in relation to the oxygen trough phenomenon. This subject has been interested in Marine Biology for a number of years and has had each summer an extended experience of relatively long periods of apnoea in the course of remaining under water to observe fish, etc.

THE ROUTE OF TRANSMISSION OF THE VIRUS OF INFECTIOUS MYXOMATOSIS OF RABBITS TO THE CENTRAL NERVOUS SYSTEM

by CHARLES SWAN[1]

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(Accepted for publication 21st February, 1941.)

In studies on the distribution of the virus of infectious myxomatosis in various organs at different periods after intratesticular inoculation, Hurst (1937c) noted that virus either appeared first in the lumbar region of the spinal cord, or was present at this level in higher concentration than elsewhere in the neural axis. These results, which suggest transmission of the virus by neural pathways, were somewhat unexpected, as Hurst (1937b) had shown earlier that this virus possesses only slight neurotropic affinities. Accordingly, the present investigation was undertaken.

MATERIALS AND METHODS.

Infected testes and spleens obtained from rabbits killed five days after intratesticular inoculation served as a source of virus. They were preserved at -10^{5} C. in 50 p.c. glycerine in normal saline solution, buffered by phosphates to neutrality. For intradermal inoculation a 10 p.c. suspension was used in doses of 0·2 c.c. to rabbits of about 1,500 gm. For intravenous administration similar suspensions were prepared with normal rabbit serum (in order to prevent intravascular clotting) and were injected in doses of 1 c.c.

TABLE 1.

Distribution of the virus of infectious myxomatosis of rabbits in nervous system following intradermal inoculation into left foot.

No. of rabbit.	No. of days after inoculation.	Anterior cerebrum and olfactory bulbs.	Middle cerebrum.	Posterior cerebrum.	Brain-stem.	Cervical cord.	Dorsal cord.	Lumbar cord.	Posterior root ganglia.	Defibrinated blood.
46	3	0	0	0	0	0	0	0	N.T.	+
76	3	0	0	0	0	0	0	0	N.T.	+
130	3	10^{-1}	0	0	0	10^{-1}	0	0	0	+
53	4	10^{-3}	10^{-1}	10^{-2}	0	10^{-2}	10^{-1}	10^{-2}	N.T.	+
64	4	10^{-2}	0	0	10^{-1}	10^{-1}	10^{-2}	0	N.T.	+
65	4	10^{-3}	0	10^{-1}	0	0	0	0	N.T.	+
127	4	10^{-3}	10^{-1}	10^{-2}	0	10^{-2}	0	0	10^{-3}	+
139	4	10^{-1}	10^{-1}	10^{-1}	0	0	10^{-2}	10^{-2}	10^{-2}	+
57	5	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-4}	N.T.	+
77	5	10^{-3}	10^{-2}	10^{-1}	10^{-1}	10^{-1}	0	0	N.T.	+
131	5	10^{-3}	10^{-4}	10^{-2}	10^{-4}	10^{-2}	10^{-2}	10^{-3}	10^{-3}	+

+ = virus was present, but concentration was not determined.
0 = no virus detected. N.T. = no test made.

All operative procedures were performed under ether anaesthesia. Denervation of the dorsum of the foot was effected by division of the femoral nerve at the level of the inguinal ligament and of the sciatic nerve at the greater sciatic foramen.

At autopsy a specimen of blood withdrawn from the right ventricle was defibrinated. The vascular system was then washed out with three litres of normal saline, according to the technique

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council, Australia.

of Hurst (1936). Representative portions of the nervous system were removed. From each a 10 p.c. (10^{-1}) suspension was prepared and centrifuged at 3,000 r.p.m. for five minutes. The concentration of virus was then determined by intradermal inoculation of serial tenfold dilutions of the supernatant in the backs of fresh rabbits. The defibrinated blood was also tested for the presence of virus, but no attempt was made to determine its titre.

EXPERIMENTAL PROCEDURE AND RESULTS.

1. *Intradermal inoculation of the virus.* Eleven rabbits were inoculated in the dorsum of the left foot, and were killed at intervals ranging from three to five days.

The results show that virus was present in the blood from at least the third day onwards. In the nervous system, on the other hand, virus was detectable in only one of three cases at the end of 72 hours, and then only in minimal amounts and in widely separated areas.

From the fourth day onwards virus was always present in the nervous system. For the most part virus appeared to be distributed at random, but the olfactory bulbs were involved more constantly than were other regions.

TABLE 2.

Distribution of the virus of infectious myxomatosis of rabbits in nervous system following intradermal inoculation into left foot three weeks after denervation had been performed.

No. of rabbit.	No. of days after inoculation.	Anterior cerebrum and olfactory bulbs.	Middle cerebrum.	Posterior cerebrum.	Brain-stem.	Cervical cord.	Dorsal cord.	Lumbar cord.	Posterior root ganglia.	Defibrinated blood.
68	3	0	0	0	0	0	0	0	0	+
116	3	10^{-2}	0	10^{-2}	0	0	0	10^{-2}	10^{-3}	+
132	3	0	0	0	0	0	0	0	0	+
26	4	10^{-2}	0	0	0	0	0	0	N.T.	+
122	4	10^{-2}	10^{-2}	10^{-2}	0	0	10^{-2}	10^{-4}	10^{-3}	+
123	4	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-3}	10^{-3}	10^{-4}	+
42	5	10^{-4}	10^{-4}	10^{-3}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	N.T.	+
103	5	0	0	0	10^{-2}	0	0	10^{-2}	10^{-3}	+
117	5	10^{-2}	10^{-3}	10^{-2}	10^{-2}	N.T.	10^{-3}	10^{-3}	N.T.	+

+ = virus was present, but concentration was not determined.
0 = no virus detected. N.T. = no test made.

TABLE 3.

Distribution of the virus of infectious myxomatosis of rabbits in nervous system following intradermal inoculation into left foot three months after denervation had been performed.

No. of rabbit.	No. of days after inoculation.	Anterior cerebrum and olfactory bulbs.	Middle cerebrum.	Posterior cerebrum.	Brain-stem.	Cervical cord.	Dorsal cord.	Lumbar cord.	Posterior root ganglia.	Defibrinated blood.
15	3	0	0	0	0	0	0	0	0	+
19	3	0	0	0	0	0	0	0	0	+
21	3	0	0	0	0	0	0	0	0	+
3	4	10^{-2}	10^{-2}	10^{-1}	0	0	0	0	N.T.	+
4	4	10^{-1}	0	0	0	0	0	0	N.T.	+
6	4	10^{-3}	10^{-1}	10^{-3}	0	10^{-1}	0	0	N.T.	+
10	4	10^{-4}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-3}	10^{-4}	N.T.	+
13	5	10^{-2}	10^{-2}	0	10^{-1}	0	0	10^{-1}	N.T.	+
18	5	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-2}	10^{-4}	10^{-3}	N.T.	+
20	5	10^{-4}	10^{-3}	10^{-5}	10^{-5}	10^{-4}	10^{-5}	10^{-4}	N.T.	+

+ = virus was present, but concentration was not determined.
0 = no virus detected. N.T. = no test made.

The level of the spinal cord corresponding to the site of inoculation was not affected prior to other levels of the neural axis. Indeed, in half of the cases the lumbar cord remained free from virus. There was therefore no evidence of spread of the virus by neural pathways.

2. *Intradermal inoculation of the virus into a denervated area.* Nineteen rabbits were injected with virus in the dorsum of the left foot. In nine of these animals denervation of the foot had been effected three weeks before. In the remainder three months had elapsed. The results are embodied in Tables 2 and 3.

It will be seen that the injection of virus into denervated skin produced no significant alteration either in the time elapsing between inoculation and the involvement of the nervous system, or in the distribution of the virus. Spread of the virus from the periphery to the neural axis by means of axis-cylinders would appear to have been eliminated.

3. *Intravenous inoculation of the virus.* The results obtained after intravenous injection showed no difference from the preceding except that more levels of the neural axis were involved at one and the same time, and the olfactory bulbs were not affected more often or more severely than elsewhere in the nervous system. They will be dealt with in greater detail in an article to be published shortly (Cooke, Hurst and Swan).

DISCUSSION.

In the present investigation, all of the evidence (the appearance of the virus in the bloodstream before it was detectable in the nervous system, the random distribution of virus in the latter, the fact that the level of the spinal cord corresponding to the area of inoculation was not the initial site of invasion of the virus, and the observation that the virus travelled equally readily from a denervated area) leads to the conclusion that the virus, when inoculated in a peripheral site, travels to the nervous system by the bloodstream and not by neural pathways.

The mechanism whereby virus reaches the central nervous system from the bloodstream may now be considered. In studies on the pathogenesis of equine encephalomyelitis, Hurst (1936) suggested that virus circulating in the blood appears on the nasal mucosa, whence it travels by the perineural lymphatics of the olfactory nerves to the subdural space. This hypothesis would serve to explain only a small proportion of the present cases.

Alternatively, Hurst (1936) suggested that the process might be a slow "growth through" the haemato-encephalic barrier, a theory which has found strong support in the recent work of King (1938, 1939). King has shown that when some strains of the virus of equine encephalomyelitis are inoculated peripherally, they enter the bloodstream and produce their initial effect on the walls of the blood vessels. The nervous system is involved secondarily.

In view of the fact that the virus of infectious myxomatosis of rabbits may produce remarkable proliferative and degenerative changes in the walls of the blood vessels (Hurst, 1937a) it is reasonable to assume, taking into account the evidence of the present study, that this virus behaves similarly to that of equine encephalomyelitis. In other words, the virus of infectious myxomatosis of rabbits, when injected peripherally, enters the bloodstream and invades the nervous system at random through the walls of the vessels.

SUMMARY.

The facts presented suggest that the virus of infectious myxomatosis is distributed to the central nervous system by means of the bloodstream.

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THE BREAKDOWN OF HEXOSE MONOPHOSPHATES IN LIVER PULP

by P. FANTL AND CHARLOTTE M. ANDERSON[1]

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(Accepted for publication 11th April, 1941.)

Fantl *et al.* (1940) have shown that in rabbit's liver pulp, incubated aerobically at 37° C., glycogen is converted partly to glucose and, to a lesser extent, to hexose monophosphate even in the presence of high concentrations of inorganic phosphate either alone or together with NaF. The suggestion was made that under such conditions the liver phosphatase concerned with the breakdown of hexose monophosphates was not completely inhibited. To test the validity of this assumption, experiments have been carried out on liver pulp to which hexose monophosphates have been added.

EXPERIMENTAL.

Reaction mixtures were prepared by adding to one part of fresh liver pulp from fasting rabbits one volume of phosphate buffer of pH 7·2, w ch contained a weighed amount of H.M.P., with (series b) or without (series a) M/10 NaF. In experiments with M/4 phosphate, the rabbits were fasted for 48 hours. To remove residual glycogen, the liver pulp was incubated for 1 hour at 37° C. before the addition of H.M.P. The potassium salt of glucose–1–phosphate is to some extent precipitated with glycogen, hence initial glycogen determinations were made in pulp without the addition of glucose–1–phosphate. The course of the reaction was followed by estimations of glycogen, inorganic phosphate and glucose during 2 hours' incubation at 37° C.

As in previous experiments, glucose was determined by Somogyi's fermentation technique. In aqueous solution the reducing glucose–6–PO_4 does not interfere with the estimation of glucose, but as a further control, the method was checked using boiled liver juice containing a known amount of glucose–6–PO_4. Here again only glucose was fermented.

The results of a series of experiments are shown in Table 1.

The increase in inorganic phosphate in the experiments 1(a)–3(a) without NaF would indicate a 100 p.c. breakdown of added H.M.P. (P × 5·8 = H.M.P. as glucose), but the glucose figures show a considerably smaller conversion, i.e. on the average 79 p.c. This discrepancy can be explained from the results of experiment 4 (a) (no NaF), in which, although no additions of H.M.P. were made, an increase in inorganic P occurred, due to the breakdown of phosphoric esters of the liver. Controls in experiments 1–3 without additions of H.M.P. have not been carried out, therefore only the glucose figures in the above experiments give a true idea of H.M.P. breakdown. From Table 1 it will be seen that hexose monophosphates are broken down nearly to the same extent, even in the presence of NaF, whereas in a control experiment No. 4 (b) with NaF, an inhibition of ester breakdown as shown by the decrease in P occurred. Thus it must be assumed that liver phosphatases behave differently towards various phosphoric acid esters.

The results of experiments 5–10 were obtained from liver pulp containing M/4 phosphate; again the added H.M.Ps. were converted to glucose and phosphoric acid, to approximately the same extent as in weak buffer solution. With the higher concentrations of inorganic phosphate the following reaction might proceed to the right:

$$\text{Glucose–1–PO}_4 \rightleftharpoons \text{Glycogen} + \text{PO}_4'''.$$

(Cori's phosphorylase system)

Here an increase in PO_4 of similar magnitude to that produced by phosphatase acting on glucose–1–PO_4 would be found, but in addition glycogen synthesis would occur. The figures do not indicate any such synthesis and we therefore believe that the liver phosphatase concerned with H.M.P. breakdown is not affected even by M/4 phosphate plus M/10 NaF under these experimental conditions.

In further experiments, the results of which are shown in Table 2, phosphatase activity was studied in liver pulp containing 0·6 p.c. Na-glycerophosphate, B.D.H. (equivalent to 56 mg. p.c. P). The usual reaction mixtures were prepared from liver pulp obtained as in the foregoing experiments. Only traces of glycogen were present.

[1] Working under a full-time grant from the National Health and Medical Research Council.

TABLE 1.

Expt. No.	Initial glycogen.	Reaction mixture.	Addition of H.M.P. calculated as glucose.	Time, hours.	Increase of glucose minus glycogen.	Increase of readily estimable P.	P.e. breakdown of H.M.P. calculated from glucose figures.
1	26	M/45 phosphate, pH 7·2	(a) 480 K salt of glucose–1–PO_4	1	300	72	62
				2	340	85	71
			(b) 480 do.	1	375	32	78
				2	435	50	91
2	400	do.	(a) 520 do.	1	235	52	45
				2	370	71	71
			(b) 520 do.	1	295	10	57
3	40	do.	(a) 200 hexose–6–PO_4 + 400 glucose–1–PO_4	1	305	97	51
				2	485	100	81
			(b) do.	1	430	51	71
				2	500	75	83
4	120	do.	(a) Nil	1	—	28	—
				2	—	38	—
						Decrease	
			(b) Nil	1	—	22	—
				2	—	31	—
						Increase	
5	70	M/4 phosphate, pH 7·2	(a) 480 glucose–1–PO_4	1	390	—	81
				2	415	—	86
			(b) 480 do.	1	350	—	73
				2	415	—	86
6	60	do.	(a) 480 do.	1	335	40	70
				2	380	40	79
			(b) 480 do.	1	300	30	62
				2	385	80	80
7	50	do.	(a) 570 glucose–6–PO_4 + 88 glucose–1–PO_4	1	370	74	56
				2	470	118	71
8	50	do.	(a) 480 glucose–1–PO_4	1	310	60	65
				2	320	90	66
9	60	do.	(a) 400 glucose–1–PO_4 + 170 glucose–6–PO_4	1	315	34	55
				2	375	90	66
10	50	do.	(a) 510 glucose–1–PO_4 + 200 glucose–6–PO_4	1	445	150	62
				2	475	192	67
			(b) do.	1	440	120	62
				2	520	126	73

Figures are in mg. per 100 gm. reaction mixture. Hexose monophosphate figures are expressed as glucose. (a) Without NaF. (b) With NaF.

TABLE 2.

	Time in hours.	Phosphate concentration. M/4	M/6	M/8
		Alteration in directly estimable phosphate. Mg. per 100 gm. reaction mixture.		
Without NaF	1	− 72	− 18	+ 12
	2	− 76	0	+ 30
+ M/10 NaF	1	− 64	− 10	+ 8
	2	− 84	− 18	+ 22
Control without glycerophosphate	1	− 76	0	− 12
	2	− 76	0	− 4

These experiments demonstrate the differential behaviour of liver phosphatases. Thus, under conditions where H.M.P. would be to a large extent broken down (Expts. 1 and 2), the phosphate values show that the breakdown of glycerophosphate is completely inhibited. With M/8 phosphate, liver phosphatase begins to exert some action.

Because of the importance of the hexose monophosphates as intermediates in carbohydrate metabolism, their behaviour in liver preparations has been studied by several workers. Cori, Cori and Schmidt (1939) found that purified liver phosphatase split glucose-1-phosphate into fermentable sugar and inorganic phosphate, and that the formation of free glucose in liver extracts containing M/20 PO_4 buffer was not inhibited by M/5 NaF. Colowick, Welch and Cori (1940) observed that hexose-6-phosphate added to an aqueous extract of rabbit's liver containing M/50 NaF is dephosphorylated under anaerobic conditions. This phenomenon, however, is masked by a re-esterification under aerobic conditions. Finally Ostern, Herbert and Holmes (1939) have reported a rapid dephosphorylation of Cori ester and Embden ester in liver brei, and state that this process was inhibited by M/200 NaF. The reaction product in the case of Cori ester was Embden ester. Our experiments with liver pulp, either in M/45 PO_4 or in M/4 PO_4 buffer, indicate that added hexose monophosphate is split into inorganic PO_4 and fermentable sugar, a process which is not influenced by M/10 NaF. The observation that liver phosphatase cannot be completely inhibited even in M/4 phosphate concentration alone or together with NaF explains why glycogen is broken down in rabbit's liver under the latter conditions for the major part to free glucose and to a lesser extent to hexose monophosphate. Under similar conditions the breakdown of glycerophosphate is completely inhibited.

It has been noted by various investigators that hydrolysis rates vary considerably with different esters. Folly and Kay (1935) pointed out that alkaline phosphomonoesterase has a much greater activity for phenyl phosphate than for glycerophosphate, which agrees with the fact that the former is hydrolysed more rapidly than the latter, the reason being that the more strongly acid the phosphoric ester, the greater will be the affinity of the enzyme for it (King and Delory, 1939). The apparent dissociation constant (pK'_2) of the substituted phosphoric acid would therefore be a decisive factor in affinity to the enzyme system.

pK'_2 of glucose-1-phosphate is 6·13 (Cori *et al.*, 1937) whereas pK'_2 of glycerophosphate is 6·34 (King and Delory, 1939). It seems therefore that glucose-1-phosphate, which is a stronger acid than glycerophosphoric acid, has a greater affinity for the phosphatase system and the link between the glucose phosphates and the phosphatase system cannot be severed by inorganic phosphate and NaF.

The Preparation of Hexose Monophosphates.

(a) Glucose-1-phosphate. In the earlier stages of this work, the process of Kiessling (1938) was used. Since several workers (Cori, 1938; Kiessling, 1939; Euler and Bauer, 1939) have shown that phosphorylation of glycogen by muscle extracts is not specific for glycogen but can occur with any polysaccharide having α-glucoside linkages, we used starch for the preparation of the two monophosphates.

7·5 gm. starch were dissolved in 200 ml. boiling water. The cooled paste was incubated for 15 minutes with 450 ml. rabbit's muscle extract after the technique of Kiessling. Glucose-1-phosphate was prepared as the barium salt (yield 11·5–12 gm.). Usually it was not possible to convert it into the crystalline potassium salt because of contamination with glucose-6-phosphate. To obtain a pure crystalline potassium salt, 10 gm. of Ba salt were heated with 50 ml. N/5 NaOH for 15 minutes in boiling water. This process destroys all the hexose-6-phosphate whereas glucose-1-phosphate as a hemi-acetal is unaltered. After centrifuging, the supernatant fluid was collected and the process repeated twice with 20 ml. quantities of N/5 NaOH. In the pooled supernatant fluids about 2 gm. of barium acetate were dissolved and then two volumes of alcohol

added. The barium salt thus separated was found to be almost pure glucose–1–phosphate, which could now be converted into the crystalline K-compound.

Analysis of the salt gave the following values:

	Found.	Theoretical. Calculated from $C_6H_{11}O_9P.K_2.2H_2O$.
Immediate reduction (Hagedorn-Jensen)	Nil	
Reduction after 10 mins. hydrolysis with N/1 H_2SO_4	48 p.c. glucose	48·3 p.c.
Directly estimable PO_4	Hardly detectable	
PO_4 after 10 mins. hydrolysis	8·2 p.c.P	8·3 p.c.

Optical rotation: $[\alpha]_D^{23} = +76{\cdot}5°$ $[\alpha]_{Hg}^{23} = +96{\cdot}7°$. Hanes (1940) gives $[\alpha]_D^{21} = +76{\cdot}7°$.

Calculating for the water-free salt from our values we have $[\alpha]_{Hg}^{23} = +107°$, and Kiessling (1938) gives $[\alpha]_{Hg}^{20} = +106{\cdot}7°$.

(b) Hexose-6-PO_4. Although several methods have been published for the preparation of hexose-6-phosphates, using either yeast press juice, glucose and phosphate (Warburg and Christian, 1932), or dialysed muscle extracts, glycogen and phosphate (Ostern *et al.*, 1936), we find it more convenient to prepare hexose-6-phosphate from undialysed muscle extract and starch, since we have seen that the conversion of glucose-1-phosphate to the 6-isomers is not inhibited by concentrated phosphate solution.

Using the same amounts of reagents as in the case of the 1–phosphate, but with an incubation time of 45 minutes, 10 gm. of barium salt were obtained. Analyses of the barium salt indicated that it is a mixture of glucose–6–phosphate, fructose–6–phosphate and probably mannose–1–phosphate. In addition approximately 5 p.c. of glucose–1–phosphate was present. Owing to the action of the isomerase of Lohmann (1933), which is contained in muscle extracts, the first formed glucose–6–phosphate is converted into the two other isomers.

Analysis of a sample of the barium salt (dried in vacuo) gave the following values: Initial reduction (Hagedorn and Jensen) 31·5 p.c. hexose; reduction after 10 mins. hydrolysis in N . H_2SO_4, 36·6 p.c. hexose; McLeod and Robison (1929) aldose titration, 18·65 p.c. calculated as glucose; fructose determined by Roe's technique (1934), 5·1 p.c.; inorganic PO_4, 0·4 p.c.; organic P after ignition, 6·32 p.c.

Since Smythe (1937) has shown that different samples of hexose–6–phosphates contain up to 15 p.c glycerophosphate, it was necessary to test for this compound. The glycerol content of the above samples of barium salts was not higher than 1·1 p.c. The determination was carried out in Pregl's micro-alcoxyl apparatus, using the volumetric technique of Vieböck and Brecher (1930). The suitability of the method was tested on sodium glycerophosphate (B.D.H.). The sample was found to contain 28·2 p.c., 28·4 p.c. glycerol by this method. Analysis of the sample gave P, after ignition, 9·3 p.c.; titration with N/2 HCl and methyl orange, equivalent weight 318. Thus, calculated as $C_3H_7O_6P\ Na_2 6H_2O$, M = 324·1, P = 9·6 p.c., glycerol = 28·4 p.c.

Note. In all the experiments of our previous paper the liver pulp was prepared by mincing through fine bronze gauze. In view of the fact that Cu^{++} inhibits phosphorylase activity (Cori, 1940) and also Robison ester formation (Gill and Lehmann, 1939), it might be argued that traces of metal went into solution, thus poisoning the phosphorylase system. Experiments were therefore repeated except that the pulp was prepared by grinding through cotton gauze.

The results were analogous to those shown in our previous paper, namely, that in spite of the high concentration of inorganic phosphate and additional NaF, glycogen is still converted for the most part into glucose, the rest being mainly glucose–6–phosphate.

SUMMARY.

Glucose-1-phosphate and hexose-6-phosphate added to rabbit's liver pulp are split into their components in both M/45 and M/4 phosphate buffer to nearly the same extent, the process being not appreciably affected by M/10 NaF. The breakdown of sodium glycerophosphate is completely inhibited in M/4 phosphate buffer.

Methods are described for the preparation of glucose-1-phosphate and hexose-6-phosphate, using fresh muscle extract and starch.

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EXPERIMENTS ON THE SURVIVAL OF RICKETTSIAE IN CELL-FREE MEDIA

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Recently reports (Findlay and MacCallum, 1940; Ellis and Spizizen, 1940) have appeared concerning the growth of different viruses in the absence of multiplying cells. These observations demand reconsideration of the statement that pathogenic viruses or bacteriophages can multiply only in the presence of actively multiplying cells.

In work with the Rickettsiae of classified European typhus and Rocky Mountain spotted fever which I carried out some years ago, and from recent investigations on *R. burneti*, indications were obtained that these organisms may grow or at least survive for a considerable time in the absence of living cells. The following is a brief report on this work.

European typhus and Rocky Mountain spotted fever. Experiments were carried out elsewhere with influenza virus (W.S. strain) and the Rickettsiae of European typhus and Rocky Mountain spotted fever. After the Rickettsiae were adapted to tissue-culture conditions by cultivation on the tunica vaginalis of guinea-pigs, they were cultivated on various organs of embryonic guinea-pigs in a 1: 3 serum-Tyrode mixture. The organs tested were tongue, lung, trachea, spleen, omentum, skin and placenta. Passages were done at fortnightly intervals and in cultures of lung and tongue the Rickettsiae grew to the third passage. No multiplication was obtained with other organs.

The influenza virus could be grown readily on the cultures with lung, and cultivation could be continued for at least ten passages at weekly intervals.

The result of these preliminary experiments favoured the hypothesis that the different viruses depend for their multiplication on some specific products of certain organs. In an attempt to cultivate viruses on artificial cell-free media, consideration was given to the fact that certain bacteria require specific substances. Thus SH compounds are essential for the growth of *B. tularensis*, and it was decided to test the influence of sulphur-containing compounds in cell-free media.

A strain of Rocky Mountain spotted fever, previously cultivated for seventeen consecutive passages on the tunica vaginalis of guinea-pigs in serum-Tyrode mixtures, was inoculated into the following media:

(1) Guinea-pig serum, 1 c.c. Tyrode's solution, 1 c.c. 1 p.c. glutathione (G.S.H.) solution, 1 c.c.

(2) Guinea-pig serum, 1 c.c. Tyrode's solution, 1 c.c. 1 p.c. sodium-thioglycolate, 1 c.c.

(3) Guinea-pig serum, 1 c.c. Tyrode's solution, 2 c.c.

In all instances two series of tests were made, one at 35° C., the other at 37° C. Transfers were made after two weeks.

Even after the first fortnight, samples 2 and 3 were free from virus if tested on guinea-pigs. In the serum-glutathione solutions the virus could be propagated for five consecutive subcultures by inoculating three drops of the previous passage into the following one. The final dilution was approximately 1/320,000.

Many subsequent attempts with different strains of Rickettsiae of European typhus and of Rocky Mountain spotted fever gave similar results in so far that the virus could be detected by guinea-pig experiments up to the third to the fifth subculture. Several attempts to improve the medium by adding different substances thought to be advantageous were not successful.

Experiments with Q Fever.[1]

Rickettsiae obtained from the spleens of infected guinea-pigs were cultivated on guinea-pig spleen serum-Tyrode mixtures at 35° and 37° C. for one week. Considerable difficulties were

[1] These experiments were carried out at the Baker Research Institute.

encountered in obtaining a "take" in the first series of cultures. The history of the strains was as follows:

	No. of Cultures.	"Takes."
1st Transfer	10	1
2nd „	5	1
3rd „	5	3
4th „	5	3

From the fifth subculture onwards three to five "takes" in each transfer were the rule. The strain could be cultivated for a total of 19 consecutive subcultures. Attempts at transfers to the different media described below were made with the fourth, tenth and sixteenth transfers. No differences were observed in strains which had been established for varying periods in tissue-culture.

(1) Cultivation in old tissue. Guinea-pig spleen was kept for 24–48 hours in the refrigerator and then used in guinea-pig serum-Tyrode mixtures in the usual way. Result: No propagation of virus in the second passage.

(2) Cultivation in serum-Tyrode mixtures previously incubated with guinea-pig spleen for one week without addition of fresh tissue. Result: Virus present in the second transfer; no virus present in the third subculture.

(3) Cultivation in Tyrode extracts of spleen without addition of fresh tissue. Result: Virus present in the first subculture; no propagation in the second.

(4) Cultivation with tissue and Rickettsiae separated by semi-permeable membranes. In these experiments guinea-pig spleen was covered with serum-Tyrode mixtures in wide test tubes. A second narrower tube closed at the bottom by cellophane membranes of different porosity and filled with serum-Tyrode mixture was placed in the larger one with the cellophane membrane in contact with the tissue. The cellophane membranes were treated according to the procedure of Seymour (1940). The Rickettsia emulsion was placed in the smaller tube. Results: No virus present in the second transfer cultivated using untreated cellophane. Virus present to the third transfer when membranes treated with 100 gm. $ZnCl_2$ or 115 gm. $ZnCl_2$ to 100 c.c. of solution were employed. No further propagation of the Rickettsiae was possible.

(5) Cultivation with tissue extracts. The same arrangement as described above was used. Tyrode extracts from guinea-pig spleen prepared by repeated grinding, freezing and thawing were placed in the wider tube and changed every week. Result: Propagation of virus was possible to the third week over fresh extracts or over extracts aerated for several hours in the cold to oxidize the tissue glutathione. (The extracts gave a negative nitro-prusside test after 2 hours' aeration.)

SUMMARY.

The propagation of Rickettsiae of European typhus and Rocky Mountain spotted fever is possible in cell-free media enriched with glutathione, and in the case of Q fever in serum-Tyrode mixtures incubated with guinea-pig spleen for one week. If tissue and Rickettsiae were separated by semi-permeable membranes of certain porosity, the same limited multiplication took place. The same phenomenon was observed when fresh Tyrode extracts of spleen were placed on one side of a similar membrane and the Rickettsiae were inoculated into serum-Tyrode solutions on the opposite side.

These results seem to indicate that several "metabolites" are necessary for the multiplication of virus or Rickettsiae. One of them is apparently easy to separate from the cells, and diffuses through membranes of a pore-diameter of approximately 1 mμ. This substance alone is not sufficient for the indefinite propagation of these organisms. If it is permissible to draw conclusions from experiments performed partly with typhus and partly with Q fever, sulphydryl compounds are necessary for the propagation of Rickettsiae, but cannot be the only necessary factor present in the tissue or in the tissue extracts because the same propagation over a limited number of passages was observed when the glutathione in the extracts was oxidized by aeration.

Acknowledgment. I wish to thank Dr. F. M. Burnet for the strain of Q fever used in these experiments.

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STUDIES ON THE HORMONAL ANTIGENS OF THE ANTERIOR PITUITARY GLAND

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(Accepted for publication 3rd April, 1941.)

Comparative serological studies of the anterior pituitary gland hormones have been made possible by the use of a simple ammonium sulphate precipitation for the isolation of various fractions. This procedure minimizes the risk of alteration of the carrier substances.

The problems discussed in this paper are: (i) The serological reactions of different hormonal fractions in relation to physico-chemical properties; (ii) The occurrence of identical or similar antigens in organs without hormonal activity; (iii) The importance of the carbohydrate component in respect of antigenicity; (iv) Whether a specific antibody against the "diabetogenic hormone" can be demonstrated.

METHODS.

Preliminary experiments showed that prolactin, growth hormone, thyrotropic hormone and follicle-stimulating hormone prepared by the method of Fevold *et al.* (1940) gave very poor antibody responses. Therefore the three antisera used were prepared as follows:

Antiserum I. Crude saline extract of anterior pituitary gland, in amounts increasing from 0·2 to 2·0 gm. fresh gland, was administered subcutaneously to rabbits on three consecutive days and the treatment repeated after four days' interval.

Antiserum II. Daily subcutaneous injections of prolactin, prepared after Lyons (1937), in amounts increasing from 1 to 20 mg. during 6 weeks. Following the maximum response, titre was maintained by two weekly injections of 20 mg.

Antiserum III. The "D" fraction of Chance *et al.* (1939) was injected daily during 6 weeks, in amounts increasing from 5 to 20 mg. Titre maintained as above.

In testing the sera, complement fixation was used in preference to precipitation methods which did not give clear cut results, particularly with the diabetogenic and crude saline antigens.

The following antigens were prepared (their abbreviated designations being shown in brackets):

1. (a) Prolactin (P)
(b) Thyrotropic hormone (TH)
(c) Growth hormone (GH)
(d) Follicle-stimulating hormone (FSH) } Prepared after Fevold *et al.* (1940).
2. Pseudo-globulin or "diabetogenic" fraction (Diab.) (Young, 1939).
3. Crude saline extract (CSE).
4. "D" fraction of Chance ("D").
5. Non-specific types prepared from ox kidney by the methods used in Nos. 1, 2 and 3 supra. (In the Tables these fractions are also referred to in short terms; thus, kidney GH refers to a kidney extract prepared in a similar manner to the growth hormone.)
6. Carbohydrate fractions: "A" substance of Landsteiner and Chase (1936). Forssmann antigen (FA) (Landsteiner and Levene, 1927).
7. Acetone soluble (AcS) and alcohol soluble (AlS) fractions prepared from dried anterior pituitary gland.

All antigens were used in a 1 p.c. normal saline solution and diluted in twofold steps.

Absorption of Sera: Absorption of the antibodies against ox serum was performed either by the methods of optimal proportion (Dean and Webb, 1926) or with alcohol-precipitated serum (Hallmann, 1939). With the hormonal fractions these methods were unsatisfactory and the antigen was absorbed on "Norit" charcoal and used for the absorption of the sera by a method analogous to that used with particulate antigens.

Biological Assay: Owing to limited facilities only thyrotropic and follicle-stimulating fractions were assayed. For assay of thyrotropic hormone the method of Rowland and Parkes (1934) was used. As an estimation of follicle-stimulating activity the smallest dose which gave a response when injected four times within 48 hours into infantile mice was taken. In the titration of the sera, injections of hormone and the anti-serum were made subcutaneously on opposite sides

of the animals. The amounts corresponding approximately to 1 unit were: thyrotropic fraction, 3 mg.; follicle-stimulating fraction, 1 mg.

In amounts up to 20 mg. prolactin was free from thyrotropic and follicle-stimulating activity. Thyrotropic hormone and growth hormone were free from follicle-stimulating action in the same amount.

The results obtained in complement fixation tests with various antigens are shown in Table 1.

TABLE 1.

Serum absorbed with	Antigen.	Complement fixation tests. Antigen dilution 0	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
		Antiserum I.								
—	CSE	±	+++	+++	+++	+++	+++	+++	+++	±
Ox serum	„	±	+++	+++	+++	++	+	+	—	—
—	P	++	+++	+++	++	+	—	—	—	—
Ox serum	„	++	+++	++	+	±	—	—	—	—
—	GH	+	+	++	++	++	+	—	—	—
Ox serum	„	—	—	+	++	+	±	—	—	—
—	TH	+	+	++	+++	+++	+	—	—	—
Ox serum	„	—	—	+	++	++	+	—	—	—
—	FSH	—	±	+	—	—	—	—	—	—
Ox serum	„	—	—	—	—	—	—	—	—	—
—	Diab.	+	++	+++	+++	+++	+++	+	—	—
Ox serum	„	—	++	+++	++	++	±	—	—	—
Ox serum	AcS	—	±	±	+	±	—	—	—	—
Ox serum	AlS	+	+	—	—	—	—	—	—	—
		Antiserum II.								
—	CSE	++	+++	+++	+++	+	—	—	—	—
Ox serum	„	—	—	+	+++	+++	±	—	—	—
—	P	+++	+++	+++	+++	+++	+++	+	—	—
Ox serum	„	++	+++	+++	++	+	±	—	—	—
—	GH	+	++	++	+	+	—	—	—	—
Ox serum	„	—	±	±	—	—	—	—	—	—
—	TH	+	++	++	±	—	—	—	—	—
Ox serum	„	—	±	++	++	+	—	—	—	—
—	FSH	—	+	++	+	—	—	—	—	—
Ox serum	„	—	+	+	—	—	—	—	—	—
—	Diab.	++	++	++	+	—	—	—	—	—
Ox serum	„	—	±	±	—	—	—	—	—	—
Ox serum	AcS	—	—	—	—	—	—	—	—	—
Ox serum	AlS	±	±	—	—	—	—	—	—	—
		Antiserum III.								
—	CSE	+	++	+++	++	++	+	—	—	—
Ox serum	„	—	—	+	++	++	—	—	—	—
—	"D"	+++	+++	+++	+++	+++	+++	+++	+	—
Ox serum	„	++	++	+++	+++	+++	+++	++	—	—
—	P	—	+	++	+	—	—	—	—	—
Ox serum	„	+	++	+	—	—	—	—	—	—
—	GH	++	++	++	+	—	—	—	—	—
Ox serum	„	—	—	—	—	—	—	—	—	—
—	TH	—	+	+++	++	++	+	—	—	—
Ox serum	„	++	++	++	+	—	—	—	—	—
—	FSH	+	+	+++	+++	+++	+++	+	—	—
Ox serum	„	+	+++	+++	+++	++	—	—	—	—
—	Diab.	+	++	+	—	—	—	—	—	—
Ox serum	„	+	±	—	—	—	—	—	—	—
Ox serum	AcS	±	—	—	—	—	—	—	—	—
Ox serum	AlS	±	±	—	—	—	—	—	—	—

There is a very poor development of anti-follicle-stimulating antibodies in the serum against crude saline extract, although this serum (against the crude extract) had a definite pro-gonadotropic action. It is anomalous that, whilst the serum against the crude extract shows a distinct reaction with the fraction containing the growth hormone, neither the serum against prolactin nor that against the "D" fraction, which between them contain the growth hormone, gives a positive reaction with growth hormone after absorption with ox serum. On the other hand, the reactions of the other hormonal fractions with anti-prolactin and anti-"D" sera are only slightly weakened by absorption with ox serum. It is peculiar that all the antibodies against growth hormone are absorbed by ox serum. From observations that will be reported later it seems that the antigen of the growth hormone fraction is similar to some antigen in ox serum, and that in preparation of the purified hormonal fraction even a moderate degree of denaturation destroys serological specificity.

TABLE 2.

Antiserum to crude saline extract.

No.	Serum absorbed with	Antigen.	Complement fixation tests. Antigen dilution 0	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
1	—	CSE	+++	+++	+++	+++	+++	++	+	—	—
2	—	P	++	+++	+++	+	+	±	—	—	—
3	P (10 mg.)	„	+	+	—	—	—	—	—	—	—
4	P (20 mg.)	„	—	—	—	—	—	—	—	—	—
5	Kidney P (2 × 20 mg.)	„	+	++	+	—	—	—	—	—	—
6	GH (2 × 20 mg.)	„	+	++	++	±	—	—	—	—	—
7	TH (2 × 20 mg.)	„	+	++	++	+	—	—	—	—	—
8	FSH (2 × 20 mg.)	„	+	+++	+++	++	±	±	—	—	
9	—	GH	—	+	++	++	+	—	—	—	—
10	GH (10 mg.)	„	—	—	—	—	—	—	—	—	—
11	P (2 × 20 mg.)	„	—	+	+	+	—	—	—	—	—
12	TH (2 × 20 mg.)	„	—	+	+	+	±	—	—	—	—
13	FSH (2 × 20 mg.)	„	—	+	+	±	—	—	—	—	—
14	Kidney GH (2 × 20 mg.)	„	—	—	—	—	—	—	—	—	—
15	—	TH	+	+	+++	++	±	±	—	—	—
16	TH (10 mg.)	„	—	—	—	—	—	—	—	—	—
17	GH (2 × 20 mg.)	„	—	+	+	+	—	—	—	—	—
18	P (2 × 20 mg.)	„	+	+	++	++	+	±	—	—	—
19	FSH (2 × 20 mg.)	„	+	+	+	+	—	—	—	—	—
20	Kidney TH (2 × 20 mg.)	„	+	++	+	—	—	—	—	—	—
21	—	Diab.	++	+++	+++	+++	+++	+++	+	—	—
22	P2(2 × 20 mg.)	„	+	++	+	±	—	—	—	—	—
23	"D" (2 × 20 mg.)	„	+	+	++	+	+	—	—		
24	P + "D" (2 × 20 mg. ea.)	„	+	++	++	+	+	—	—	—	—
25	P + "D" (2 × 20 mg. ea.)	„	+	+	++	+	±	—	—	—	—
26	—	CSE	+++	+++	+++	+++	++	±	—	—	—
27	"A" (2 × 20 mg.)	„	+	+++	+++	+	±	—	—	—	—
28	"F" (2 × 20 mg.)	„	+++	+++	+++	++	+	—	—	—	—
29	"A" + "F" (2 × 20 mg. ea.)	„	+	+++	++	+	±	—	—	—	—
30	P + "D"	„	+	++	++	+	+	—	—	—	—
31	No. 30 absorbed with "A" + "F" (2 × 20 mg. ea.)	„	+	++	++	+	—	—	—	—	—
32	—	AcS	+	+	—	—	—	—	—	—	—
33	—	Als	+	++	++	+	—	—	—	—	—
34	"D" (2 × 20 mg.)	„	+	++	±	—	—	—	—	—	—
35	AlS (2 × 20 mg.)	CSE	++	+++	+++	+++	++	++	—	—	—
36	„ „	Diab.	+++	+++	+++	+++	++	+	—	—	—
37	„ „	"D"	++	+++	+++	++	+	—	—	—	—
38	„ „	P	+	+++	+++	+	—	—	—	—	—

Tables 2, 3 and 4 show the results of tests with sera absorbed with different anterior pituitary or non-specific fractions. The anti-sera were firstly absorbed with ox serum and then with the particular fraction to be tested. Absorptions were carried out with 10 or 20 mg. If antibodies still persisted, a fresh batch of serum was treated with two successive quantities of 20 mg. (2 × 20 mg.). If no decrease in reaction was observed between the absorption tests with 20 mg. and 2 × 20 mg., only the latter result is given.

TABLE 3.

Anti-prolactin serum.

No.	Serum absorbed with	Antigen.	Complement fixation tests. Antigen dilution 0	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
1	—	P	+++	+++	+++	+++	+++	+++	+	—	—
2	P (10 mg.)	„	—	—	—	—	—	—	—	—	—
3	GH (2 × 20 mg.)	„	+++	+++	+++	+++	+	—	—	—	—
4	TH (2 × 20 mg.)	„	+++	+++	+	+	±	—	—	—	—
5	FSH (2 × 20 mg.)	„	+++	+++	+++	+++	+++	+	—	—	—
6	CSE (2 × 20 mg.)	„	++	+++	+++	+	—	—	—	—	—
7	CSE (2 × 20 mg.)	„	+	+++	+++	±	—	—	—	—	—
8	Kidney P (10 mg.)	„	+	+	++	+	—	—	—	—	—
9	Kidney P (2 × 20 mg.)	„	—	—	—	—	—	—	—	—	—
10	Kidney TH (2 × 20 mg.)	„	++	+++	+++	+++	+	—	—	—	—
11	Kidney GH (2 × 20 mg.)	„	++	+++	+++	+++	+++	+	—	—	—
12	"A" (2 × 20 mg.)	„	++	+++	+++	++	—	—	—	—	—
13	"A" + FA (2 × 20 mg.)	„	+	+++	+++	++	+	—	—	—	—

TABLE 4.

Anti-"D" serum.

No.	Serum absorbed with	Antigen.	Complement fixation tests. Antigen dilution 0	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
1	—	"D"	+++	+++	+++	+++	+++	+++	+	±	—
2	"D" (10 mg.)	„	+	+++	+++	+	—	—	—	—	—
3	"D" (20 mg.)	„	—	—	—	—	—	—	—	—	—
4	—	TH	+	+++	+++	+	+	—	—	—	—
5	GH (10 mg.)	„	+	+++	+++	+	+	—	—		
6	GH (20 mg.)	„	+	++	++	±	—	—	—		
7	GH (2 × 20 mg.)	„	+	+	±	—	—	—	—		
8	FSH (10 mg.)	„	+	+++	+++	+	+	—	—	—	—
9	FSH (2 × 20 mg.)	„	+	+++	+++	+	±	—	—	—	—
10	—	FSH	+	+++	+++	+++	+	—	—	—	—
11	TH (20 mg.)	„	—	+	+	+	—	—	—	—	—
12	TH (2 × 20 mg.)	„	—	±	±	—	—	—	—	—	—
13	Kidney "D" (2 × 20 mg.)	"D"	++	+++	+++	++	—	—	—	—	—
14	Kidney TH (2 × 20 mg.)	TH	+	+++	++	—	—	—	—	—	—
15	Kidney FSH (2 × 20 mg.)	FSH	—	+	+	—	—	—	—	—	—

These results indicate that the different hormone fractions have some antigenic component in common. There is a close relationship between hormones isolated by similar extraction and precipitation methods.

The antibodies against the growth hormone can be easily absorbed by the specific antigen or by approximately four times the amount of non-specific kidney growth hormone fraction (Table 2, Nos. 10 and 14).

Similar tests with crude saline extract antiserum and anti-"D" serum show that the prolactin, thyrotropic and follicle-stimulating fractions behave differently. The strengths of the reactions can be decreased by about one-half (Table 2, No. 5; Table 4, Nos. 14 and 15), but complete absorption of the antibodies is impossible. On the other hand, the antibodies against prolactin in anti-prolactin serum (Table 3, No. 9) can be completely removed by the corresponding fraction from ox kidney.

These findings suggest that even the simple and apparently non-denaturing method of isolation by repeated ammonium sulphate precipitation has altered the antigenic behaviour of the hormonal fractions. Finally, it seems that the thyrotropic and follicle-stimulating hormones are serologically closely related. Almost all the antibodies against the follicle-stimulating fraction can be absorbed with thyrotropic hormone (Table 4, No. 12), but only a very slight lessening of the reaction with the thyrotropic hormone can be produced by absorption of the serum with follicle-stimulating hormone (cf. Table 4, Nos. 8 and 9). This seems to indicate that an antigen very similar to that of the follicle-stimulating hormone occurs in the thyrotropic hormone, but in addition another is present which is unrelated to the follicle-stimulating hormone and is responsible for specificity of the thyrotropic hormone.

Importance of carbohydrate components. Some of the methods for isolation of different hormone fractions are very similar to those employed for the isolation of antigenic carbohydrate fractions. It was thought, therefore, that carbohydrate components could play a part in the antigenicity of the hormone fractions. All sera gave positive complement fixation tests with both Forssman antigen and "A" substance, but absorption of the sera with those substances did not show decrease in titre with the hormonal preparations. Antigens similar to Forssmann antigen and "A" substance can be said to occur therefore in pituitary fractions, but they are in no way related to the fractions containing the true hormones.

In the previous experiments it was sometimes observed that antisera produced against various fractions of the anterior pituitary gland gave different reactions with the same antigen. Furthermore, reports occur in the literature that various hormone fractions can be isolated in a non-antigenic state, which casts some doubt on the specificity of the antibodies at present under investigation. To investigate the importance of "non-antigenic" fractions, prolactin and thyrotropic fractions, as used in the experiments reported in the previous tables, were precipitated with 60 p.c. alcohol and the precipitate dried with ether. The biological potency of the hormone preparations was unchanged by this treatment. The results obtained in complement fixation experiments are reported in Table 5. This shows that the so-called loss of antigenicity is a physical phenomenon, since the antigenicity could be restored by absorption on collodion particles.

TABLE 5.

Complement fixation tests.

No.	Serum.	Antigen.	Antigen dilution: 0	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
1	Anti-prolactin	P	+++	+++	+++	+++	+++	+++	+	—	—
2		After one precipitation with alcohol	+++	+++	++	+	—	—	—		
3		After two precipitations	—	±	±	—	—	—	—		
4	Anti-"D"	TH	+++	+++	+++	++	±	±			
5		After two precipitations with alcohol	++	+++	+	—	—	—			
6		After three precipitations	+	+	±	—	—	—			
		Complement fixation with Nos. 3 and 6 after addition of collodion particles.									
7	Prolactin	3	++	+++	+++	+++	+++	+++	++	—	—
8	Anti-"D"	6	+++	+++	+++	+	—	—	—	—	—

Since antibodies with a true protective action have been observed against a number of pituitary hormonal fractions, it is of considerable interest to know whether the diabetogenic hormone has an antigenic specificity of its own. The fact that the antibodies against the diabetogenic pseudo-globulin fraction cannot be absorbed with the various purified fractions shown in Table 6 lends weight to this suggestion. It must, however, be kept in mind that failure to exhaust the antibodies against the pseudo-globulin fraction might be due to partial denaturation during purification of the various fractions. A definite conclusion cannot be reached until a biological test has been devised.

TABLE 6.

Serum No. 1 absorbed firstly with ox serum, then with substances as shown.

No.	Serum absorbed with	Antigen.	Complement fixation tests. Antigen dilution 0	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
1	Ox serum	CSE	+++	+++	+++	+++	+++	++	±	—	—
2	„ „	Diab.	+	+++	+++	+++	+	—	—	—	—
3	Diab. (20 mg.)	CSE	+	++	++	++	+	—	—	—	
4	„ „ „	P	—	—	—	--	—	—	—	—	—
5	„ „ „	TH	—	—	+	++	+	—	—	—	—
6	„ „ „	GH	—	+	++	—	—	—	—	—	—
7	TH + GH (2 × 20 mg. ea.)	Diab.	—	++	+++	++	—	—	—	—	—
8	P (2 × 20 mg.)	„	—	++	+++	+	—	—	—	—	—
9	"D" (2 × 20 mg.)	„	—	++	+++	++	—	—	—	—	—
10	"D" + P (2 × 20 mg. ea.)	„	—	++	+++	++	—	—	—	—	—
11	Albumin*	„	—	++	+++	++	—	—	—	—	—
12	Albumin + Euglobulin† (2 × 20 mg. ea.)	„	±	+	++	+	—	—	—	—	—
13	AcS + AlS (2 × 20 mg. ea.)	„	+	+	+++	+++	+	—	—	—	—
14	Kidney pseudo-globulin (2 × 20 mg.)	„	±	+++	+++	+	—				

* Albumin fraction from CSE of anterior pituitary gland.
† Euglobulin fraction from CSE of anterior pituitary gland.

Biological tests. A few biological tests were carried out in order to check the serological results. The main points of interest in such a comparison are: (1) The possibility of progonadotropic action of a serum against crude pituitary extract. This serum did not contain antibodies against the purified follicle-stimulating fraction. (2) The biological confirmation of the serological observation that all reaction of the anti-"D" serum with the follicle-stimulating fraction disappears after absorption with the thyrotropic hormone fraction, whilst the reaction with the thyrotropic hormone is not decreased following absorption with the follicle-stimulating hormone. (3) The confirmation of the observation that the strength of the reaction of the different hormone fractions decreases to an appreciable extent following absorption of the sera with an analogous fraction from ox kidney.

TABLE 7.

Pro-gonadotropic action of serum 1 (anti-CSE).

Amount of "D" fraction injected.	Serum.	Result.
7·5 mg.	—	3 +
5·0 mg.	—	2 + 1 —
2·0 mg.	—	1 + 2 —
1·0 mg.	—	1 + 2 —
0·5 mg.	—	3 —
1·0 mg.	1 c.c.	3 +
0·5 mg.	1 c.c.	1 + 2 —
0·25 mg.	1 c.c.	1 + 2 —

The results of these experiments are illustrated in Tables 7, 8 and 9. In the case of pro-gonadotropic and anti-gonadotropic action three mice were used for each test, a positive follicular response being indicated by a + sign, a negative by a — sign.

These results confirm the suggestion of Rowlands (1939) that the pro-gonadotropic action of certain anti-hormonal sera might be caused, at least partially, by their action on fractions unrelated to the hormone proper.

TABLE 8.

Anti-gonadotropic action of serum III (anti-"D").

Amount of follicle-stimulating hormone injected.	Serum.	Result.
5 mg.	—	3 +
5 mg.	1 c.c.	1 + 2 —
5 mg.	1 c.c.*	3 +
5 mg.	1 c.c.†	3 +

* Absorbed with 3 treatments each of 20 mg. of thyrotropic fraction.
† Absorbed with 3 treatments each of 20 mg. of kidney FSH.

TABLE 9.

Anti-thyrotropic action of serum III (anti-"D").

Amount of thyrotropic hormone injected.	Serum.	Increase in weight of the thyroid gland.
3 mg.	—	21 mg.
5 mg.	—	36 mg.
3 mg.	5 c.c.	10 mg.
3 mg.	5 c.c.*	23 mg.
3 mg.	5 c.c.†	18 mg.

* Absorbed with 3 × 30 mg. kidney TH.
† Absorbed with 3 × 30 mg. FSH.

These experiments confirm the conclusions which have been drawn from the serological reactions.

DISCUSSION.

In serological experiments with the advantage of improved technique in so far as the antigens are concerned, it has been possible to confirm the observation that many protein fractions of the anterior pituitary gland have specific antigens closely correlated with their hormonal actions. As in the case of the hormonal fractions proper, the antigenic properties of these protein fractions are very sensitive to denaturing influences. This is so marked that even following simple ammonium sulphate precipitation distinct differences are readily discernible. This is particularly well illustrated in the case of the growth hormone which does not produce specific antibodies if partially purified and also with prolactin, where the antibodies directed against this fraction could be removed from the anti-prolactin serum with an analogous fraction from ox kidney but not from the serum against crude extract.

All the hormonal fractions have a common antigen which is more marked in some instances, e.g. the case of the thyrotropic hormone, which has the same antigen as the follicle-stimulating hormone in addition to having an antigen of its own. This latter is not found in the follicle-stimulating hormone, and to this is due the specificity of the thyrotropic hormone. It is improbable that this is due to an admixture of follicle-stimulating hormone with thyrotropic hormone, since the latter was found to be free from follicle-stimulating action. It is also highly improbable that the thyrotropic hormone contains an admixture of follicle-stimulating hormone in an inactivated form, because all hormones were prepared by the same method of simple ammonium sulphate precipitation.

Similar, if not identical, antigens were found in analogous fractions prepared from ox kidney.

The fact that fractions prepared from the anterior pituitary gland and kidney have similar antigenic properties on the one hand, but differ on the other hand in that the latter produce no hormonal effects, indicates that the structural differences must be very slight.

In addition to the antigens of the well-defined hormonal fractions, other antigens occur in the pituitary gland, one of them in the pseudo-globulin fraction, which contains the diabetogenic hormone. Because of the lack of a suitable biological test this antigen could not be correlated with the postulated diabetogenic hormone. Further studies concerning this problem are in progress.

It has been shown that some carbohydrate and lipoid components of the pituitary gland, which are antigenic, exert no influence on the antibody formation of the hormonal fraction.

SUMMARY.

The antigenic properties of hormonal fractions isolated from pituitary glands were compared with analogous fractions from ox kidney. The same antigens were found in both. The significance of these observations is discussed.

Observations are presented on antigens other than those associated with the known hormonal fractions and on the influence of carbohydrate and lipoid components on the antigenicity of the pituitary gland.

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STUDIES IN THE EPIDEMIOLOGY OF Q FEVER

8. THE TRANSMISSION OF Q FEVER BY THE TICK *RHIPICEPHALUS SANGUINEUS*

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As a further step in determining the range of potential vectors of Q fever, transmission experiments were performed with *Rhipicephalus sanguineus* Latreille (1804), the common brown dog tick of Queensland.

In May, 1940, engorged female ticks were collected from kennels at the Brisbane Dog Refuge. A laboratory stock was readily established and maintained using guinea-pigs as hosts. No evidence of the presence of any agent infectious to guinea-pigs was obtained by examination of ticks of this stock; no host animals became infected, no rickettsiae were seen in smears of ticks, and no ticks infected guinea-pigs into which they were inoculated.

1. *The infection of larval, nymphal and adult ticks.* Ticks at all stages of development were successfully infected with Q fever by feeding them upon infected guinea-pigs during the febrile period.

Larval ticks were fed upon infected guinea-pigs two to three weeks after hatching. The ticks engorged within three to four days. Nymphal ticks were likewise exposed to infection two to three weeks after ecdysis. They engorged and were removed from their hosts after four to seven days of attachment. These larval and nymphal ticks were not examined prior to their next ecdysis, but subsequent examinations, which will be described later, revealed that many of them had acquired infection.

Six partially-replete female ticks with attendant males were placed upon infected guinea-pigs to complete their engorgement during the febrile period of the host animals. At the completion of oviposition, fifteen to twenty-one days after engorgement, the virus was recovered from five ticks by means of guinea-pig inoculation. Smears prepared from these ticks prior to their inoculation contained, in three instances, abundant rickettsiae. Numerous rickettsiae were seen in the sixth female tick which was examined by means of serial sections. Although rickettsiae were not seen in smears of the male ticks, a guinea-pig into which they were inoculated became infected with Q fever.

2. *Transfer of the virus from larva to nymph.* The presence of the virus in nymphal ticks, developed from larvae which fed upon infected guinea-pigs, was proved by the demonstration of rickettsiae in stained smears of their tissues and the production of infection in guinea-pigs into which they were inoculated or upon which they were fed. (Table 1.)

TABLE 1.

Transfer of the virus from larva to nymph.

Examination of Unfed Nymphs.			Feeding of Nymphs.			
Days after ecdysis.	Rickettsiae seen in smears.	Result of guinea-pig inoculations.	Days after ecdysis.	Ticks per guinea-pig.	Number of host guinea-pigs.	Result.
1	1*	‡ [1]	2	(8)	1	Transmission not effected.
	10†	§ [1]	8–10	(2); 8; (11); 14;	12	Nine guinea-pigs infected.
17	1*	‡ [1]		14; 20; 21; 22;		
	7†	§ [1]		24; (31); 33; 34;		
30	5*	‡ [5]	17	5; (5); (6);	3	One guinea-pig infected.
	2†	§ [1]				

* Rickettsiae seen. † No rickettsiae seen. ‡ Animals infected. § Animals not infected. [ ] Number of guinea-pigs inoculated. ( ) Number of nymphs which fed upon the animals which did not become infected.

Twenty-six unfed nymphs were examined by smears for the presence of rickettsiae. Seven of them were found to be infected. Rickettsiae were seen in one of eleven ticks examined on the first day after ecdysis, in one of eight examined on the seventeenth day, and in five of seven examined on the thirtieth day. Each of these ticks produced infection in the guinea-pig into which it was inoculated. Animals inoculated with the remaining ticks did not become infected.

Nymphal ticks were fed in batches, numbering from two to thirty-four ticks, upon guinea-pigs at intervals ranging from two to seventeen days after ecdysis. The ticks were removed replete after five to seven days. Ten of sixteen host animals become infected. The incubation periods (after the removal of the ticks) in nine instances ranged from six to thirteen days. The tenth animal had an inapparent infection which became obvious on passage.

3. *Transfer of the virus from nymph to adult.* The transference of the virus from nymphal to adult ticks was proved by the examination of smears and sections and inoculation and feeding tests.

Examination of smears and inoculation of guinea-pigs. One hundred and sixty-six adult ticks, previously exposed to infection during either their larval or nymphal stadium, were examined separately after feeding as adults upon guinea-pigs. Seventy-eight ticks were removed from their hosts for examination either prior to, or at the onset of, the fever induced by their presence; fifteen of them were dissected for the preparation of smears prior to their inoculation into separate guinea-pigs; the remainder were tested for infection by the examination of either smears or serial sections. The data relevant to the examination of these ticks are summarized in Table 2. Forty-nine ticks (63 p.c.) were proved to be infected. No significant difference existed between the percentages of infected male and female ticks.

TABLE 2.

Transfer of the virus from nymph to adult.

Examination of adult ticks.

Mode of examination.	Number of ticks examined.	Number of ticks with visible rickettsiae.	Number of guinea-pigs infected.	Number of infected ticks.
Smears	40	29		29
Smears and guinea-pig inoculation	15	8	9	9
Serial sections	23	11		11
Totals	78			49

The remaining eighty-eight ticks, all of them females, were permitted to remain upon their hosts until completely engorged; the majority of them fed during all or part of their hosts' period of fever. Approximately 80 p.c. were shown to be infected by the examination, after oviposition, of a single smear taken from a caecum of the gut.

Feeding of adults. Infected adult ticks were fed at intervals ranging from one to three weeks after ecdysis upon thirty-eight guinea-pigs, all but three of which became infected. (Table 3.)

TABLE 3.

Transfer of the virus from nymph to adult.

Feeding of adults.

Days after ecdysis.	Number of of ticks placed on each guinea-pig.	Number of guinea-pigs.	Number of guinea-pigs which became infected.	Remarks.
8	1 ♂ 1 ♀	1	1	The ♂ tick only was infected.
19	3 ♂ 3 ♀	5	5	All ticks were infected.
13	6 ♂ 6 ♀	12	11	Approx. 63% of these ticks would be infected.
15	6 ♂ 6 ♀	20	18	
Totals	208 ♂ 208 ♀	38	35	

Upon each of five animals which became infected were fed three male and three female infected ticks; a sixth animal was infected by a single male tick.

Upon each of the remaining thirty-two animals were placed six male and six female ticks. The exact number of infected ticks which fed upon each of these guinea-pigs was not known, as

many of them were examined only after engorging during the period of their hosts' acquired infection. Twenty-nine of these guinea-pigs became infected. The majority of animals reacted with fever seven to nine days after the ticks were placed on them. The shortest period was six days and the longest nineteen days. Infected ticks fed upon each of the three animals which failed to become infected.

4. *Failure of transovarial passage.* A representative number (200–300) of the progeny of each of one hundred and thirteen female ticks were examined for evidence of transovarial passage of the virus. Eighty-three of these females were known to be infected. It is probable that many of the remainder, which had been exposed to infection either as larvae or nymphs and again as adults, were also infected.

The progeny of batches of ten females were ground up together and inoculated into guinea-pigs. Three animals were inoculated each with the progeny of a single infected tick. No evidence of the presence of the virus in the larval ticks was obtained.

5. *Distribution of rickettsiae within the tick.* Twenty-four adult ticks were examined by means of serial sections. One female tick which had engorged upon a febrile guinea-pig was found to be infected. Of the other twenty-three ticks, which had been exposed to infection during either their larval or nymphal stadium, four of twelve females and seven of eleven males contained rickettsiae.

In the infected ticks, all of which were examined after feeding, the lumen and lining epithelium of the midgut and its diverticula were packed with rickettsiae. No rickettsiae were seen in situations other than these. In infected cells rickettsiae were seen only in the cytoplasm; invasion of the nuclei was never observed.

Faeces collected from adult ticks during their engorgement were found to be highly infectious. Castaneda-stained smears contained large numbers of rickettsiae per field, and titration tests made sixty-five days after collection revealed the minimum infecting dose for guinea-pigs to be 10^{-8} gm.

6. *Preparation of rickettsial suspensions suitable for agglutination tests.* Infected ticks were found to provide an excellent source of rickettsiae for the preparation of suspensions suitable for agglutination tests. Female ticks treated after oviposition according to the technique of Freeman *et al.* (1940) yielded about 3 ml. of rickettsial suspension per individual.

DISCUSSION.

In Australia the cosmopolitan dog tick *Rhipicephalus sanguineus* has become established in Queensland and the Northern Territory; its range probably extends into the northern districts of New South Wales. There are no records of its occurrence in Victoria, South Australia or Western Australia. First recorded from Queensland by Neumann (1897), it is now widespread throughout the State. Roberts (1935) records its occurrence upon sheep, cattle, the horse, cat and man as well as upon its principal host the dog.

In the Mediterranean Basin *R. sanguineus* is known to be the natural hereditary vector of two rickettsial diseases; fièvre boutonneuse (Blanc and Caminopetros, 1931; Combiesco, 1932), and canine rickettsiosis (Donatien and Lestoquard, 1936). The tick has been considered a potential vector of Rocky Mountain spotted fever in the eastern and south-eastern United States of America since Parker and his associates (1933) demonstrated experimental transmission and generation to generation passage of the virus.

In view of the experimental findings and the occurrence of the tick in Queensland upon domestic animals which are known or potential reservoirs of infection, it may be regarded as a potential vector of Q fever.

SUMMARY.

The tick *Rhipicephalus sanguineus*, which occurs in northern Australia upon domestic animals which are known or potential reservoirs of infection, may be regarded as a potential vector of Q fever.

Larval, nymphal and adult ticks were infected with Q fever by feeding them upon infected guinea-pigs during the febrile period.

The virus was passed from larvae to nymphs and from nymphs to adults, but not from the latter to their progeny.

Infected nymphs and adults infected their host guinea-pigs in 63 p.c. and 92 p.c. of cases respectively.

In infected ticks rickettsiae were seen only in the lumen and lining epithelium of the midgut. Invasion of the nuclei of infected cells was not observed.

One-hundred-millionth of a gram of faeces from infected ticks was found, sixty-five days after collection, to infect guinea-pigs.

Suspensions of rickettsiae suitable for agglutination tests were readily prepared from infected ticks.

Acknowledgments. I am indebted to Sir Raphael Cilento, Director-General of Health and Medical Services, Queensland, for permission to publish this paper; also to Mr. Collins Greaves for his technical assistance; and Officers of the C.S.I.R. and the several State Departments of Agriculture for information regarding the Australian distribution of the tick.

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THE EFFECT OF OXIDIZING AGENTS ON HAEMOCYANIN

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The reversible combination of haemocyanin with oxygen has led many workers to conclude that it has a chemical structure essentially similar to that of haemoglobin, and attempts have been made to show that the former pigment possesses a range of derivatives analogous to those of the latter, in particular, compounds with carbon monoxide or containing bivalent instead of the supposed univalent copper and also a prosthetic compound containing a much higher content of copper than haemocyanin. Work already published from this Institute (Rawlinson, 1940a) has thrown doubt on the existence of a carbon monoxide compound and of a separate prosthetic group. In this paper are described experiments which suggest that, in the case of the haemocyanin of *Jasus lalandii*, the oxidizing agents used by other workers (Conant, Chow and Schoenbach, 1933) do not alter the pigment, and that the explanation of the results described in the paper referred to must be sought elsewhere.

Preparation of Reagents.

Crystalline haemocyanin. The preparation and purification of the crystalline haemocyanin have been described in detail (Rawlinson, 1940a).

Potassium molybdicyanide. This compound, $K_3Mo(CN)_8$, which was very sensitive to light, was prepared and successfully stored for several months. A detailed investigation into the magnetic properties of this complex in its reduced and oxidized states was also described (Rawlinson, 1941). All work with this oxidizing agent was carried out either in the dark or in red light.

Experiments with Oxidizing Reagents.

The methods used in the attempts to oxidize *Jasus* haemocyanin followed closely those described by Conant and collaborators. The concentration of haemocyanin solutions was between 5 and 10 p.c. and the oxidizing solutions were never greater than 0·01 N., to reduce protein destruction to a minimum. The actual amounts of oxidizing agents were varied from two up to six equivalents in excess of the copper present. The calculation (Conant *et al.*) on the assumption that the permanganate was reduced to the manganous state was considered unsound because in neutral solution it was doubtful whether reduction occurred below the quadrivalent state. After the addition of oxidizing agents the solutions were allowed to stand so that chemical reaction might be complete. The solutions were then dialysed against water and their purification was attempted. The samples treated with larger amounts of reagents were more difficult to crystallize, particularly those in which permanganate was used. In such cases purification was possible by precipitation with ammonium sulphate. The majority of samples were found to crystallize when isoelectric buffer solution (acetate–pH 4·6) was added. Samples of each preparation were examined for the presence of manganese or molybdenum compounds respectively and it was invariably found that the crystals were less contaminated than the fine amorphous precipitate obtained by the use of ammonium sulphate. The purified samples were washed well with water and dissolved in dilute ammonia solution. By this means it was possible to obtain solutions containing up to 20 p.c. of treated pigment and these were compared directly with similar concentrations of the original pigment.

Magnetic Measurements.

The aim of this work was to confirm the existence of a haemocyanin derivative which contained copper in the cupric state. Magnetic measurements of many copper compounds have shown that the cuprous state is diamagnetic whilst the cupric state is paramagnetic. The magnetic moment of the latter state corresponds to one unpaired electron (theoretical moment of 1·73 Bohr magnetons). Unlike several transition-group elements no details of the bond type are possible from such magnetic data in the case of copper. The experiments recorded in Table 1 were designed less to give an absolute value for the magnetic susceptibility of the protein than to detect

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a difference between samples of the original pigment and of the "oxidized" pigment under similar conditions. Any change in susceptibility would suggest a change of valence of the copper. The reagents which were considered necessary for these experiments are among the highest oxidation systems at present known. For magnetic work potassium permanganate is not permissible since the products of its reduction possess greater paramagnetism than the copper of the pigment. The only substances available are either potassium tungsticyanide or potassium molybdicyanide. The latter substance was used in this work; it possesses a magnetic moment of 1·66 Bohr magnetons (Rawlinson, 1941) and on reduction becomes diamagnetic.

TABLE 1.

Magnetic measurements on haemocyanin solutions.

No.	W_s	W_w	W_h	D_s	D_w	D_h	C_h	$\chi \cdot 10^6$	Notes
1	0·5875	0·4692	0·1183	— 32·00	— 27·00	— 5·00	20·14	— 0·529	C.O.
2	0·5877	0·4694	0·1183	— 31·93	— 27·01	— 4·99	20·14	— 0·528	C.O.
3	0·5852	0·4735	0·1117	— 31·91	— 27·24	— 4·66	19·08	— 0·523	C.O.
4	0·5851	0·4734	0·1117	— 31·88	— 27·25	— 4·63	19·08	— 0·520	C.O.
5	0·5851	0·4719	0·1132	— 31·92	— 27·14	— 4·78	19·34	— 0·528	O.P.
6	0·5864	0·4730	0·1134	— 31·92	— 27·21	— 4·71	19·34	— 0·520	O.P.
7	0·5848	0·4717	0·1131	— 31·88	— 27·13	— 4·75	19·34	— 0·526	O.P.
8	0·5854	0·4722	0·1131	— 31·93	— 27·16	— 4·77	19·34	— 0·527	O.P.

W_s = weight of solution in tube (gm.), W_w = weight of water in this solution, W_h = weight of haemocyanin in this solution, D_s = deflection by solution in tube (cm.), D_w = deflection by water in this solution, D_h = deflection by haemocyanin in this solution, C_h = concentration of haemocyanin in this solution (gm. per 100 gm. solution), χ = mass susceptibility of haemocyanin. C.O. = solution of purified oxyhaemocyanin, O.P. = solution of "oxidized" pigment.

Quantitative Results.

Magnetic susceptibility of haemocyanin and "oxidized" haemocyanin. The apparatus used for the work presented in Table 1 was the modified Curie-Cheneveau torsion balance described in an earlier paper (Rawlinson, 1940b). The methods of calculation and the corrections necessary have been described (Rawlinson, 1941). The standard substance was water; $\chi = -0{\cdot}720 \times 10^{-6}$ at 18° C. and with the fibre used, 0·5545 gm. of water gave a deflection of — 31·90 cm. The average value for the magnetic susceptibility of haemocyanin was $-0{\cdot}525 \times 10^{-6}$ and for the "oxidized" haemocyanin $-0{\cdot}525 \times 10^{-6}$; the calculated value for the latter if the copper had changed in valency would be $-0{\cdot}495 \times 10^{-6}$ (on the assumption that the magnetic moment of the cupric state was 1·73 and the copper content was 0·148 p.c.).

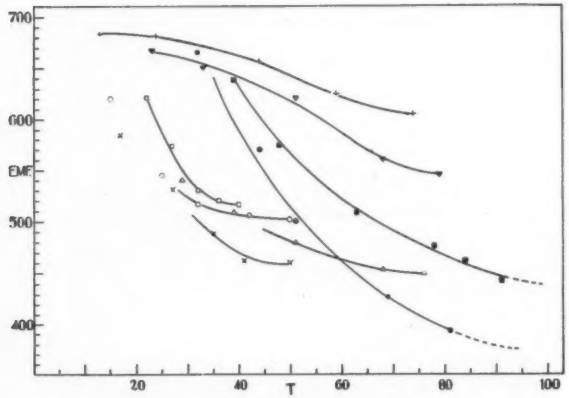

Fig. 1. Extrapolated potentials.

Abscissae: T. is time after adding a further volume of oxidant.

Ordinates: EMF. is the potential of the solution in millivolts.

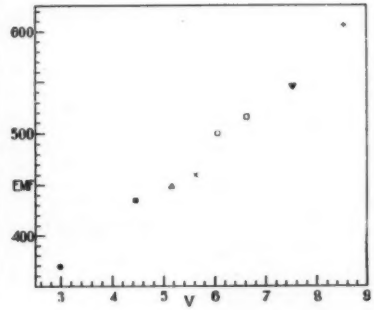

Fig. 2.

Electrometric titration results.

Abscissae: V. is volume of 0·0100 N. potassium molybdicyanide.

Ordinates: EMF. is the extrapolated potential in millivolts from Fig. 1.

Electrometric titration of haemocyanin. The work of Conant and co-workers (1933) was repeated and a typical result is represented by Fig. 1. This experiment with potassium molybdicyanide was carried out in an atmosphere of nitrogen, although the results of experiments in air were found to be similar. The development of steady potentials, after each addition of oxidant, was found to take one to two hours. The values recorded (Fig. 2) were obtained by extrapolation after measuring the potential changes over known intervals of time (see Fig. 1). These potentials were measured against a saturated calomel electrode and have not been referred to the standard hydrogen electrode. The apparatus which was described in an earlier paper (Rawlinson, 1939) served in these experiments. This electrometric titration was carried out with 15·0 c.c. of 19·6 p.c. haemocyanin and 0·0100 N. potassium molybdicyanide.

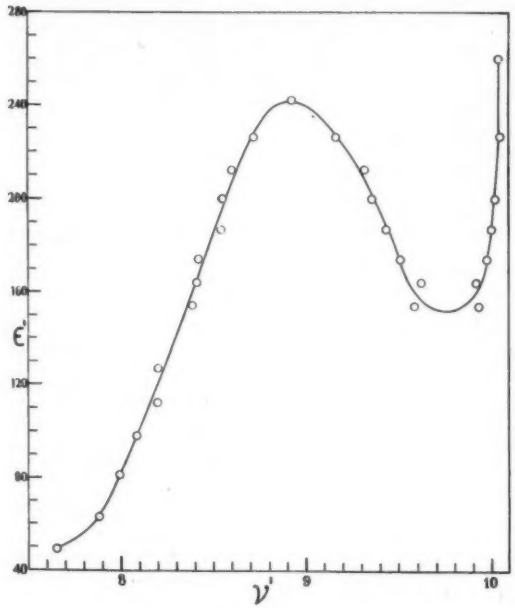

Fig. 3. Ultra-violet absorption spectrum of "oxidized" pigment.

Abscissae: $\nu^1 = \nu \cdot 10^{-14}$ where ν is frequency per sec.

Ordinates: $= \epsilon^1 \cdot 10^2$ where $\epsilon = \frac{1}{cd} \log \frac{I_0}{I}$.

c = No. of grams of substance per litre of solution. d = Depth of cell in cm. I_0 = Intensity of incident beam. I = Intensity of emergent beam.

Ultra-violet absorption spectrum of the "oxidized" haemocyanin. A Hilger echelon-cell type of spectrophotometer was used to measure the absorption of ultra-violet light by this pigment. Fig. 3 shows that the absorption band after oxygenating is in the same position as that of the original haemocyanin previously measured (Rawlinson, 1940a).

DISCUSSION.

The magnetic measurements which were performed on this copper-protein compound were designed to confirm the conclusions of Conant and his collaborators. Instead of supplying such confirmation, these results have cast a doubt on the reported oxidation. This necessitated a detailed investigation into the experiments of the above workers, and their findings will be briefly summarized and discussed.

No previous evidence had enabled a statement to be made concerning the valency of the copper in haemocyanin. The reagents which caused oxidation of haemoglobin were without effect, but three of the most powerful oxidizing agents were considered to have oxidized the copper of *Limulus* haemocyanin. The new derivative was called methaemocyanin and formed with oxygen a greenish-blue pigment (oxymethaemocyanin). Both of these substances showed oxidizing properties and combined with potassium cyanide in a manner similar to that of haemocyanin. Electrometric determinations were somewhat unsatisfactory but were deemed further evidence for the existence of such derivatives.

After a close study of the methods used by these authors it was concluded that their results were capable of other explanations. The effect of dilute potassium permanganate, on haemocyanin solutions at pH 7, will be first considered. This oxidizing agent, in neutral or alkaline solution is not reduced to the manganous state; under normal conditions, with excess of permanganate, manganese dioxide is the end-product. Potassium permanganate is reduced when added to any protein in solution and this means that some organic groups (for example, the — SH groups) of the latter are oxidized. This reduction of permanganate was observed with haemocyanin solu-

tions and Conant assumed that part was caused by the copper of the protein. The resulting solution contained ''oxidized'' protein, manganese dioxide and perhaps some manganous salt. The manganese dioxide was partially precipitated and partially held in colloidal solution by the protein. The purification of such protein solutions is considered, by the author, to be practically impossible. Rawlinson (1938) showed that heavy metals, when added to protein solutions, were very difficult to remove without causing destruction of the native protein. The removal by centrifugation of a small amount of brownish precipitate was mentioned by Conant, who also reported the presence of a small amount of brownish colour in the solution after the oxidation of haemocyanin by potassium permanganate, in an atmosphere of nitrogen. The author found, in all experiments with permanganate, that ''oxidized'' *Jasus* haemocyanin was coloured faintly brown in completely deoxygenated solutions. This was quite noticeable in the concentrated solutions employed in this work but in the dilute solutions as used by Conant it was difficult at first to appreciate that such a low intensity of brown colour was capable of making the ''oxidized'' pigment appear greenish-blue. It was this colloidal manganese dioxide which bestowed most of the oxidizing power on the ''oxidized'' pigment; in addition the oxidized organic groups probably contributed some oxidizing power. The reduction, by sodium hyposulphite, of a solution containing the greenish-blue ''oxymethaemocyanin'', was found to give rise to the blue oxyhaemocyanin on shaking with oxygen. This experiment was of no value because the ''oxidized'' pigment consisted of the blue oxyhaemocyanin contaminated with the brown manganese dioxide sol and the resulting colour was greenish-blue. The sodium hyposulphite reduced this manganese dioxide to the colourless manganous state and reoxygenation then formed the blue oxyhaemocyanin.

The qualitative tests which were used by Conant to confirm the presence of cupric copper are invalid in the presence of manganese dioxide, because the latter substance causes the development of the characteristic oxidation colours with the reagents used, i.e. gum guaiacum, reduced indophenol and benzidine sulphate.

The strongest argument brought forward by Conant and co-workers in favour of the assumption of the presence of a cupric compound is the quantitative determination of the oxidizing power of the solutions of ''methaemocyanin''; one equivalent per atom of copper was reported from titration data. While it is not easy to assign such a constant value to a colloidally protected contaminant, two facts may be noted. The first is that in spite of every effort such contamination was always present and secondly the contaminant has the properties which would enable it to produce the results reported. Their methaemocyanin solutions were about 10^{-3} normal with respect to copper and in the presence of protein a colloidal solution containing an equivalent concentration is quite possible. With restricted working conditions a constant protein concentration leads to a constant concentration of any substance peptized. The quantitative experiments on whole blood were interpreted with reserve because such solutions cause undue complications of the oxidation-reduction system.

The experiments in which potassium molybdicyanide was used may be explained in a similar manner. It was found that this reagent was difficult to remove from the protein, even after repeated crystallizations. The yellow potassium molybdocyanide therefore caused the blue oxyhaemocyanin to appear greenish-blue. In this case the oxidizing power of the protein was enhanced by potassium molybdicyanide which remained present as a contaminant. De-oxygenation, by evacuation was sufficient to show that the complex cyanides were still present with the ''purified'' protein.

The oxidation-reduction potential of the haemocyanin system will now be discussed. Experiments repeated by the author showed that steady potentials were developed only after a long time. The results which are given in Figs. 1 and 2, were obtained by extrapolation but this is obviously unsatisfactory. It must be remembered that in these oxidation-reduction experiments the organic matter was being treated with some of the most powerful oxidizing substances. Conant and his collaborators suggested that ''each small increment of oxidizing agent reacts to some extent with the organic part of the protein molecule as well as with the copper''. Under such circumstances it is not logical to assume that the cuprous-cupric system is the sole contributor to the oxidation-reduction potential. The general electrochemical equation $E = E_0 - \frac{RT}{nF} \ln \frac{[\text{oxidized}]}{[\text{reduced}]}$, does not allow us to differentiate between individual systems; the change of potential depends upon the ratios $\frac{[\text{oxidized}]}{[\text{reduced}]}$ for separate substances, at constant hydrogen-ion concentration. From molecular weight determinations the value of ''n'' was known to be large and the last term in the above equation tended to be smaller than in many less complex systems. Conant found that the potentials during the back titration of ''methaemocyanin'', with sodium hyposulphite, were considerably below those obtained during the oxidation of haemocyanin with molybdicyanide. This suggests that they were not dealing with a reversal of the cuprous-cupric system. Another system with a lower potential was apparently impressing itself upon the electrode. The potential of the oxygen electrode is higher than that of the reported haemocyanin-methaemocyanin potential but the above workers were not successful in finding a catalyst capable of activating the oxidation of haemocyanin; one possible reason for this is now clear.

The ultra-violet absorption spectrum of the greenish-blue "oxidized" pigment was found to be identical with that of the original native protein and this offers an independent argument in favour of the identity of "oxymethaemocyanin" as contaminated oxyhaemocyanin. The band which appeared on oxygenation was situated at 3355Å.

The unsatisfactory state of our knowledge concerning this respiratory protein was further emphasized in earlier studies on the so-called prosthetic group. In papers by Conant and collaborators (1933, 1934) they appeared to be convinced that a real prosthetic group existed. Rawlinson (1940a) was led to conclude that the green product, obtained by the alkali degradation of haemocyanin, was composed of copper, amino-acids and sulphur compounds (probably sulphide or amino-acids). After further study of this substance the original opinion was still held. It was considered impossible to assume that the amino-acids found in the green residue were the actual groups combined with the copper in the native protein. The copper atoms were probably separated from the protein molecule immediately after the solution was made alkaline and combination with free amino acids was considered to occur after hydrolysis of the protein. This assumption differs fundamentally from the facts known about haemoglobin derivatives. The iron atom of haemoglobin is situated at the centre of a porphyrin ring and this real prosthetic group combines with globin through the metallic atom.

I now suggest that the copper atoms in the haemocyanin molecule do not require a separate prosthetic group to activate them for this combination with the protein moiety. The absence of such a prosthetic group would allow the copper atoms more chemical bonds for direct attachment to the protein. Such extra directing linkages from the copper may also explain the ease with which many haemocyanins crystallize. The crystallization of insulin is facilitated by the presence of zinc atoms, and this offers support for the above suggestion.

By orientation of certain groups, the protein of haemocyanin probably confers the specific property, on the copper atoms, of combining with oxygen. The haemocyanins of all species so far investigated have been shown to combine with one oxygen molecule for every two copper atoms present. The blue colour of the oxyhaemocyanin might then be caused by the development of a resonating structure when each oxygen molecule is linked between two copper atoms. The valency state of the copper is still an open question, and all attempts to obtain further evidence have so far failed. Experiments in which reducing agents such as an aluminium-mercury couple were used caused no obvious change to solutions of *Jasus* haemocyanin at pH7. Further investigations into the reactions and chemical structure of complex copper compounds are necessary before this work can be clearly understood.

SUMMARY.

The conclusions which have been reached about haemocyanin derivatives are considered to be most unsatisfactory. The tendency of early workers was to search for derivatives analogous to those of haemoglobin. This fallacy was demonstrated previously in the case of carbon monoxide, and this paper presents chemical and magnetic evidence which fails to support the existence of an oxidized derivative of *Jasus* haemocyanin, such as was claimed for *Limulus* haemocyanin. It is suggested that no separate prosthetic group is present, but that specific orientation of the protein molecule enables the copper atoms to become activated for their combination with molecular oxygen; reasons are advanced for this suggestion.

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EXPERIMENTAL POLIOMYELITIS

1. THE EFFECT OF FARADIC STIMULATION ON THE LOCALIZATION OF PARALYSIS INDUCED BY THE INTRANASAL INOCULATION OF POLIOMYELITIS VIRUS

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From time to time, trauma has been suggested as an aetiological factor in the development of diseases of the nervous system. Pritchard (1934) reported a case in which during the incubation period of poliomyelitis a particular muscle of the upper limb was fatigued excessively. The subsequent paralysis showed itself most severely in the fatigued muscle, which later became completely atrophic; the remainder of the affected muscles showed complete, or almost complete, recovery. In an epidemic of poliomyelitis at a boarding school, de Rudder and Petersen (1938) found in a proportion of cases that strenuous physical exertion was a factor in increasing susceptibility to the disease. Similar observations have been made by Abercrombie (1925–26), Martin (1933), Leake (1936), van Rooyen and Rhodes (1940), and others.

Although the above reports suggested the present investigation, it must be stressed at the outset that the experimental procedures adopted in no way lead to the simulation of normal muscular fatigue. For the induction of such fatigue it would be necessary to stimulate the anterior horn cells synaptically, that is, by means of a reflex. To those with experience in the handling of monkeys, the difficulties of such a procedure, especially when the use of an anaesthetic is precluded, are obvious.

In the present paper an account is given of an attempt to determine whether faradic stimulation of the muscles of a particular limb during the incubation period of poliomyelitis might be a factor in increasing the susceptibility of the anterior horn cells to the virus and in the localization of the initial paralysis.

Apparatus used for faradic stimulation. An induction coil and a three volt dry-cell battery connected together in series were used. In addition a buzzer was connected in parallel with the induction coil.

To the secondary circuit of the induction coil two electrodes were connected. One of these, the indifferent electrode, was a plate of sheet zinc measuring 1½ by 2½ inches, and covered with eight thicknesses of cotton gauze. The other, the stimulating electrode, was a glass rod, 5 inches long, around the lower half of which copper wire had been wound spirally. Superimposed over the wire and the portion of the glass rod around which it had been wound were eight layers of cotton gauze.

The strength of the stimulus used was determined in the first place empirically. When tested on our forearms the stimulus caused marked tetanization of the muscles and a moderate degree of pain.

Actual measurement showed that the frequency of the stimulus was approximately 60 per second. The voltage output on open circuit was approximately 50 volts R.M.S., and 1,100 volts peak.

Experimental procedures. Eight rhesus monkeys were infected with poliomyelitis by the intranasal inoculation of a 10 p.c. suspension of infected spinal cord substance on three successive days. Five of these animals were stimulated while the remaining three acted as controls.

Faradic stimulation was begun on either the first or second day after the initial intranasal inoculation, and was applied for a period of half an hour per day until the onset of paralysis, when it was discontinued.

In all cases the left upper limb was selected as the site for stimulation.

Anaesthesia was induced with A.C.E. mixture and maintained with ether. The animal was placed on its back on a large rubber mat, and tied in position by means of ropes attached to

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both ankles and the right wrist. The indifferent electrode, previously moistened in saline, was then inserted between the posterior aspect of the left arm and the rubber mat, the weight of the limb ensuring adequate contact. The stimulating electrode was applied in turn to the motor points of the muscles belonging to the various groups of the arm, forearm and hand until fatigue occurred. By the time the more distal groups of muscles had been fatigued, the proximal groups, which had been tetanized earlier, showed some degree of recovery and responded to further stimulation. In the half hour period it was possible to repeat this cycle about four times.

The results are shown in the following table.

Effect of faradic stimulation on localization of paralysis.

No. of monkey.	No. of days stimulated.	Paralysis, distribution (in order of appearance) and severity.
S. 50	8	Left arm +++, right arm + and legs +.
S. 51	8	Legs +++, left arm +++, right arm ++.
S. 69	17	Right leg +, left arm +.
S. 70	9	Left arm +++ and right arm ++, legs ++.
S. 71	9	Legs +++, left arm ++, right arm +.
S. 48 (control)	0	Right leg +++ and left leg ++, left arm +.
S. 49 (control)	0	Legs +++ and right arm +++, left arm +.
S. 73 (control)	0	Legs ++, arms +++.

+++ = Severe paralysis.
++ = Moderate paralysis.
+ = Mild paralysis.

Of the five monkeys subjected to stimulation, only two, S 50 and S 70, manifested their initial paralysis in the limb which had been stimulated. In the latter animal the opposite arm was involved as well. The paralysis in the remainder of the monkeys, S 51, S 69 and S 71 began first in the lower limbs. Later, however, when the upper limbs became affected the paralysis was more severe in the arm which had been stimulated.

In the control animals, S 48, S 49 and S 73, the lower limbs were the first to become paralysed, but in S 49 the initial palsy occurred in the right arm as well. A comparison of the intensity of the paralysis of the upper limbs, shows that in one case the arms were involved equally, while in the remaining two, the left and the right arms were each involved more severely on one occasion.

In general the paralysis was not more severe in the animals subjected to stimulation. Indeed, S 69, which had been stimulated on seventeen successive days, was the least affected.

DISCUSSION.

The stimulated limb was the site of the initial palsy in only two out of five monkeys. As a similar localization of the initial paralysis occasionally occurred in other experiments in which monkeys were infected by the intranasal route, it cannot be concluded that the site of the initial palsy is influenced by electrical stimulation.

Nevertheless the paralysis in the upper limbs was in all cases more marked in the arm subjected to stimulation, suggesting that faradic stimulation increases the susceptibility of anterior horn cells to the virus. This increase in susceptibility may be the result of impulses passing antidromically along the axons to the motor cells.

SUMMARY.

Faradic stimulation of a particular limb during the incubation period of poliomyelitis did not influence the localization of the initial paralysis. In all five cases, however, the stimulated limb was paralysed more severely than its fellow on the opposite side.

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STUDIES ON THE COPPER CONTENT OF THE MILK OF SHEEP AND OF COWS

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Observations made by Bennetts and Chapman (1937) suggest that lowered copper intake in sheep causes a very marked lowering of the milk copper. This is in contrast with the effect of a dietary deficiency of other inorganic constituents (Groenewald, 1935), which causes no such lowering of the concentration in the milk. Other observers had found that feeding an excess of copper salts to cows (Elvehjem, Steenbock and Hart, 1929) and to sheep (Thomas, 1937) caused no increase in milk copper.

The copper content of milk from normal ewes and cows as determined by various workers is shown in Table 1.

TABLE 1.

Authors.	Animals.	Milk copper range. mg. per litre.
Elvehjem *et al.* (1929)	Cows	0·123–0·184
Krauss and Washburn (1934)	,,	0·14 –0·17
Grimmer (1934)	,,	0·19 –0·34
Conn *et al.* (1935)	,,	0·051–0·132
Sylvester and Lampitt (1935)	,,	0·09 –0·17
Echava (1936)	,,	0·31 –0·40
Miwa (1938)	,,	0·22 –0·37
Grimmer (1934)	Ewes	0·27 –0·29
Thomas (1937)	,,	0·46

Bennetts and Chapman (1937) found 0·12 and 0·19 p.p.m. respectively in the milk of two "normal" ewes which had run on copper deficient pastures for two months and had lambed about three weeks previously. In none of the results reproduced in Table 1 is any attention directed to a relation between the milk copper levels and the period of the lactation in which the samples were taken. The present paper is concerned with the presentation of further data concerning the variations of the copper content of the milk of normal sheep and cows and with the effect of lowered copper intake on the milk copper concentration.

Methods. The milk samples were taken directly into glass containers. The cows' milk samples were a composite of the foremilk from the four quarters and were usually taken in the afternoon. An estimation on the morning and afternoon foremilk of two cows disclosed no difference in copper concentration, nor were the strippings significantly higher than the foremilk.

A suitable quantity of the milk (50–100 ml.) was evaporated in a silica basin with 5 ml. of 20 p.c. magnesium nitrate (Cu free) per 50 ml. milk. After ashing at an incipient red heat, the ash was dissolved in hydrochloric acid and filtered on an acid-washed filter paper. The small amount of carbonaceous residue usually contained a few microgrammes of copper so the filter paper and residue were digested with 1 ml. H_2SO_4, 0·4 ml. 60 p.c. $HClO_4$ and sufficient HNO_3 to destroy organic matter and carbon. The mixture was evaporated to sulphuric fumes, cooled and diluted. Mixing this digest with the HCl extract precipitates calcium sulphate so the two solutions must be extracted separately. Both were neutralized with NH_3 and carefully brought to pH 3

with dilute HCl using brom-phenol-blue as indicator. The subsequent procedure was as described by Sylvester and Lampitt (1935); the dithizone solution used to extract the H_2SO_4–$HClO_4$ digest was also used for the original HCl solution. A blank was done on all reagents. Although somewhat lengthy, the method gives excellent results with duplicates and with recoveries of added copper.

The blood copper was estimated by the method of Tompsett (1934).

Results.

1. *The copper content of normal ewes' milk.* The first sheep tested were pure bred merinos depastured in the drier agricultural areas of Western Australia. The sheep were run on natural pasture which previously was found to have the following copper content: 10·6 p.p.m. (dry basis) on 24/7/39, 6·0 p.p.m. on 30/8/39 and 5·2 p.p.m. on 27/3/40 when the pasture was dry. Supplementary feeding with cereal hay was necessary after lambing in April until the end of June. Green feed was only available during July and August, but there was ample dry feed and both ewes and lambs remained in excellent condition throughout.

The first samples were taken ten to fifteen days after the ewes had lambed. The results of the analysis of the milk and blood at monthly intervals for a period of four months are given in Table 2.

TABLE 2.

Copper content of milk and blood of merino ewes.

(Both as mg. Cu per litre. Figures for blood in italics.)

GROUP 1: MERREDIN.

Ewe.	24/4/40.		22/5/40.		26/6/40.		31/7/40.		28/8/40.	
B193	0·63	*0·96*	0·16	*0·92*	0·11	*0·96*	—	*0·96*	0·08	*1·10*
B243	0·24	*0·89*	0·14	*0·90*	0·08	*1·13*	0·06	*1·13*	0·11	*1·29*
B261	0·43	*0·79*	0·15	*0·73*	0·08	*0·98*	0·06	*1·13*	0·08	*1·14*
V70	0·64	*0·78*	0·16	*0·83*	0·09	*0·88*	0·04	*0·93*	0·10	*0·92*
V75	0·36	*0·69*	0·16	*0·48*	0·07	*0·60*	0·04	*0·66*	0·10	*0·79*
V79	0·41	*1·18*	0·19	*0·90*	0·16	*1·05*	0·07	*1·12*	0·12	*1·44*
Mean of milk copper	**0·452**		**0·160**		**0·098**		**0·054**		**0·098**	
S.D.	±0·157		±0·017		±0·033		±0·013		±0·015	

"B" sheep four-toothed. "V" sheep full-mouth.

The second group (Table 3) consisted of seven crossbred (merino-Border Leicester) ewes from a locality of slightly better rainfall and grazing conditions where the natural pastures had a copper content of 8 to 13 p.p.m. (dry basis). Ample green feed was available from the end of June onward. As in the case of the first group, the first samples were taken about a fortnight after lambing and subsequently at monthly intervals for three months. The last samples of milk were obtained with difficulty as the ewes were drying off. Blood copper determinations were also made and are included in Table 3.

TABLE 3.

Copper content of milk and blood of cross-bred ewes.

(Both as mg. Cu per litre. Figures for blood in italics.)

GROUP 2: BEVERLEY.

Ewe.	11/6/40.		11/7/40.		6/8/40.		3/9/40.	
B4	0·53	*0·87*	0·48	*1·05*	0·14	*1·06*	0·22	*0·84*
B34	0·42	*0·97*	0·31	*1·45*	0·16	*1·10*	0·14	*1·14*
B55	0·44	*0·96*	0·36	*1·38*	0·16	*1·17*	0·21	*1·05*
B91	0·20	*0·98*	0·11	*1·25*	0·11	*1·13*	0·13	*0·94*
B112	0·48	*0·75*	0·18	*0·96*	0·14	*1·46*	0·19	*0·94*
B241	0·38	*0·92*	0·18	*1·10*	0·06	*1·10*	0·12	*1·06*
B270	0·39	*0·94*	0·25	*0·96*	0·12	*1·11*	0·17	*1·09*
Mean of milk copper	**0·406**		**0·267**		**0·127**		**0·169**	
S.D.	±0·105		±0·126		±0·035		±0·040	

B112, B241, B270, six-tooth; B4, B34, B55, full-mouth; B91, aged.

2. *The copper content of normal cows' milk.* The first group of cows (Table 4) were grazed on natural pasture, the copper content of which was 9 to 10 p.p.m. (dry basis) in the Spring months. These cows were pure bred Guernseys, mastitis-free and all details of their past history were available. During the months of January to June, the animals were on dry grazing and were receiving as a supplementary ration increasing quantities of mixed cereal silage (4·2 p.p.m. Cu dry basis) with some green lucerne and a crushed grain-concentrate mixture. From July, cereal chaff was substituted for the silage. Green feed was not available in the paddocks in any quantity until the beginning of August.

TABLE 4.

Copper content of cows' milk.

(Mg. per litre. Blood copper figures for four cows are in italics.)

GROUP 1: MURESK.

Cow No.	Weeks after calving at first test.	Weeks of test.							
Sub group A		0	2	6	10	14	16	18	22
100	44	0·04	0·05	*	—	—	—	—	—
130	40	0·05	0·03	0·05	0·05	—	0·04	—	—
70	37	0·03	0·02	0·05	*	—	—	—	—
151	36	0·03	0·03	0·05	*	—	—	—	—
86	1·5	0·38	0·18	0·10	0·06	0·08	—	—	0·07
		0·92	*0·96*	*0·86*	*1·02*	*0·85*	—	—	*1·08*
69	0·5	0·14	0·23	0·12	0·06†	0·11	—	0·07	—
Sub group B		0	1	2	3	4	5	8	12
108	8	0·06	—	—	—	0·07	—	0·06	—
167	4	0·13	—	—	—	0·11	—	0·05	—
188	4	0·10	—	—	—	0·06	—	0·11	0·07
199	1	0·12	0·17	0·27	—	0·19	0·18	0·10	0·09
		1·01	*1·02*	*1·06*	*1·01*	*1·40*	*0·93*	*0·98*	*1·10*
562W	1	0·06	0·09	0·06	0·07	0·06	—	0·06	—
		0·94	*0·93*	*0·85*	*1·00*	*1·25*	—	*0·90*	—
100	1	0·07	0·07	0·10	—	0·10	0·08	0·09	—
Sub group C		0	1	2	3	4	5	6	11
70	1·5	0·12	0·09	0·12	—	—	—	0·07	—
201	1·5	0·10	0·18	0·11	0·22	0·08	0·14	0·10	0·06
		1·00	*0·86*	*0·96*	*0·69*‡	*0·82*	*0·77*	*0·90*	*1·03*
DF117	2	0·10	0·09	0·10	—	0·07	—	—	—
151	2	0·12	0·11	0·09	—	0·07	—	—	—
Sub group D		0	1	2	3	4	6	7	8
76	2	0·23	0·16	0·16	0·13	0·11	—	—	0·07
204	1·5	0·34	0·23	0·19	0·19	0·11	—	—	0·09
210	1	0·24	0·16	0·15	0·12	—	—	0·09	—
203	1	0·29	0·29	0·19	0·25	—	0·20	—	—

* Cows drying off, see below for next lactation. † Cow sick. ‡ Tuberculin injection on previous day.

Sub groups A–C: Summer calving.

A Cows 69 and 86 calved January, 1940, others March to May, 1939.
B Calved January to April, 1940.
C Calved May, 1940.

Sub group D: Winter calving, July, 1940.

Cows arranged in order of calving.

Blood copper was estimated simultaneously with most of the milk samples. As no systematic variation occurred, the blood copper figures are given (Table 4) for only four of the cows, selected as representing the four main types of variation which occur in the milk copper figures.

The second group of cows (Table 5) were grazed on irrigated pastures, the copper content of which was from 9 to 14 p.p.m. (dry basis). A blood copper determination was done on all cows on 5/9/40; most of the figures lay within the normal range (0·87 to 1·04 mg. per litre) but six of the cows (Nos. 14, 20, 28, 42, 46 and 66) showed rather higher values (1·19 to 1·66 mg. per litre).

TABLE 5.

Copper content of cows' milk.
(Mg. per litre.)

GROUP 2: HARVEY IRRIGATION AREA.

Cow No.	Breed.*	Weeks after calving at first test.	Weeks of test.					
Sub group A			0	1	2	3	4	6
53	SXJ	3	0·05	0·04	—	—	0·05	0·04
51	J	2·5	0·06	0·04	—	—	0·05	—
36	J	2·5	0·10	0·05	—	—	—	0·05
52	SXJ	2·5	0·09	0·05	—	—	—	—
28	S	2	0·07	0·06	0·05	0·07	—	—
50	S	1·5	0·06	0·05	0·04	0·04	—	—
Sub group B			0	1	2	4	7	8
68	SXJ	1·5	0·12	0·10	0·09	—	0·08	—
66	GS	1	0·10	0·10	0·07	—	—	—
20	S	0·5	0·06	0·06	0·08	—	—	—
55	GS	0·5	0·13	0·11	0·16	0·11	0·14	0·12
Sub group C			0	1	2	3	4	
29	S	1	0·07	0·10	0·09	0·05	—	
46	S	1	0·08	0·06	—	—	0·05	
14	S	0·5	0·10	0·06	0·05	—	—	
42	S	1·5	0·13	0·13†	—	—	—	
22	GF	0·5	0·20	0·21	—	—	—	

* S, Shorthorn; J, Jersey; F, Friesian (Holstein); X, cross; G, grade.
† Circumstances prevented the continuation of the experiment beyond this date.

Calving dates: Sub group A, early June; B, early July; C, August, 1940.
Cows arranged in order of calving.

3. *The effect of decreased copper intake on the milk copper levels of sheep and cows.* Milk samples were taken from a number of experimental ewes at Gingin, a copper-deficient area in Western Australia (see Bennetts and Chapman, 1937). Three groups were available: (1) Controls grazing on copper-deficient pastures, 2–3 p.p.m. (dry basis), (2) sheep having access to a salt lick containing 0·5 p.c. copper sulphate, and (3) sheep running on pasture which had been top dressed with 20 lb. copper sulphate per acre and contained 10–12 p.p.m. copper (dry basis). Group 3 may be regarded as normal. As some of the sheep of Group 2 showed low blood copper figures, it is probable that all the animals were not taking an adequate amount of lick. The blood copper figures are included in Table 6 to give some indication of the copper status of the animals.

TABLE 6.

Milk copper of Gingin sheep.
(Mg. per litre. Blood copper figures in italics.)

Group.	Sheep No.	9/8/39.		22/8/39.		19/9/39.	
1	133	0·03	*0·15*	0·04	—	0·13	*0·59**
(Cu-deficient	234	0·02	*0·07*	0·02	*0·07*	0·02	*0·10*
pasture only)	282	—	*0·14*	0·03	*0·06*	0·01	*0·16*
	289	0·01	*0·11*	0·02	*0·09*	0·01	*0·17*
2	229	—	*0·32*	0·04	*0·28*	0·05	*0·49*
(Cu-deficient	283	—	—	0·07	*0·67*	0·10	*1·04*
pasture plus	286	0·11	*0·50*	0·07	*0·48*	0·05	*0·61*
Cu-lick)	295	0·15	*0·84*	0·11	*0·74*	0·10	*1·04*
3	249	—	*0·99*	0·11	*1·00*	0·10	*0·89*
(High Cu pasture)	255	—	—	0·09	*0·84*	0·15	*0·98*
	267	—	—	0·09	*0·44*	0·11	*0·86*
	290	—	*0·34*	0·10	*0·75*	0·09	*1·02*

* See note in text.

Sheep 290 lambed 24/6/37; Nos. 229, 234, 249, 286 between 27/6/40 and 18/7/40; others in June.

The analysis of a number of milk samples from copper deficient cows is given in Table 7. These cows were from two "Falling Disease" areas (Bennetts and Hall, 1939) and were grazing on pastures containing between 1 and 2 p.p.m. copper (dry basis).

TABLE 7.

Milk copper levels of copper deficient cows.

(Mg. per litre.)

Group A.			Group B.		
Calving date.	1/11/39.	13/12/39.	Calving date.	28/11/39.	12/12/39.
8/10/39	0·04	0·02	14/ 7/39	0·01	0·01
11/ 5/39	0·01	0·02	17/ 9/39	0·025	0·02
2/10/39	0·015	0·02	3/11/39	0·02	0·015
2/10/39	0·05	0·01	23/ 9/39	0·02	0·01

DISCUSSION.

Several facts emerge from the data for normal animals. In all cases, the concentration of copper in the blood is higher than in the milk. The mammary glands apparently have no mechanism for concentrating copper in the milk as occurs with some other inorganic constituents. Since the milk copper can vary enormously without any corresponding variation in the blood level, it is reasonable to assume that the transfer of copper from the blood to the milk is not one of passive diffusion but is subject to physiological control.

The fact that ewes and many cows give greatly increased milk copper in the early part of the lactation period, seems to have escaped the attention of other workers, and it is obvious that the stage of lactation is important when milk copper levels are considered. The Guernsey cows from Group 1 show this phenomenon more than the Shorthorns from Group 2, but the fact that sheep also show raised levels in the early lactation period suggests that it is far more fundamental than merely the effect of breed. The age of the cows apparently has no influence in deciding whether or not raised milk copper levels will occur. Although the limited data are insufficient to draw any conclusions concerning seasonal effects, it should be noted that there is a greater tendency for raised levels among cows calving in the winter months when the copper content of the pastures is higher.

The slight rise which occurs with almost all the normal sheep on the last sampling may be associated with the "drying off" of the ewes.

The values obtained for most of the copper deficient ewes confirms the original observation of Bennetts and Chapman (1937) that, under these circumstances, the milk copper may fall to very low levels. There is no obvious reason for the sudden rise of both blood and milk figures for ewe 133 on 19/9/39 (blood figures re-checked). The animal was slaughtered on 17/10/39 and its liver found to contain only 9·2 p.p.m. copper (dry basis) indicating a state of acute copper deficiency.

Unfortunately, no samples were taken from these copper deficient ewes shortly after lambing. However, the data of Bennetts and Chapman show that in such ewes the milk copper is slightly higher shortly after lambing, but still far below that of a normal sheep. The milk of three such ewes collected 1, 2 and 3 weeks after lambing contained 0·05, 0·03 and 0·05 p.p.m. respectively. This point has been confirmed by samples taken from an acutely copper-deficient ewe from another area, 0·09 mg. per litre being found two weeks after lambing.

It is apparent that under conditions of low copper intake, cows can produce milk with a copper content much below normal. Only two of the figures obtained lie within the normal range, and these are from cows within a few weeks of parturition.

SUMMARY.

An examination of the copper content of the milk of ewes and cows from normal and copper deficient areas of Western Australia has been made. It was found that:

The copper content of the milk of normal ewes fell progressively from values of 0·20 to 0·64 mg. copper per litre in early lactation to 0·04 to 0·16 mg. per litre several months later.

Similar falls were observed in the milk of some cows, but were not apparent in others. A considerable variation in the values was obtained, but the majority of the figures lay between 0·05 and 0·20 mg. copper per litre.

There was no correlation between blood copper levels and the milk copper variations.

Sheep and cows grazing on copper deficient pastures (1 to 3 p.p.m. copper dry basis) show reduced levels of copper in the milk. Values down to 0·01 to 0·02 mg. copper per litre were obtained in most cases.

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THE ACTION OF ALKALI ON THE CHEMICAL AND PHYSIOLOGICAL PROPERTIES OF ADRENALINE

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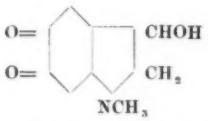
(Accepted for publication 14th March, 1941.)

In an earlier paper (Shaw, 1938) it has been shown that the reduction of arsenomolybdic acid by adrenaline is increased by the addition of alkali. This fact has been made the basis for a sensitive quantitative and a specific qualitative test for adrenaline. The present paper tells of an attempt to identify the substance formed by the action of alkali and an investigation of its physiological action.

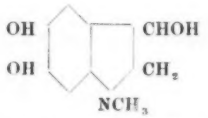
Raper and co-workers have shown that during the oxidation of tyrosine by tyrosinase a "quinonoid" intermediate is formed, which is changed later to an indole derivative. Ball and Chen (1933) postulated the fleeting formation of a quinone intermediate during the oxidation of adrenaline but did not offer any chemical evidence in support of its existence.

Welch (1934) has stated that adrenaline may undergo a reversible oxidation and reduction in the tissues and has mentioned (1935) the possibility of the protective action of ascorbic acid as being due to the inhibition of the oxidation or the reduction of any oxidized adrenaline already formed. He also suggested that the first step in the oxidation of adrenaline might be quinone formation.

Green and Richter (1937) have isolated its characteristic red oxidation product and believe it to have the formula



This substance, termed adrenochrome, contains both the indole and quinone grouping. It is unstable in alkali changing to



which is yellowish, and termed the leuco base. No doubt the red colour of adrenochrome is due to its "quinonoid" nature. Green and Richter have stated that adrenochrome is without physiological activity. The present author has found, using the frog's heart, that its effect is about 3 p.c. that of adrenaline. This is true also of the leuco base.

As preliminary experiments had shown that the substance responsible for the increased reducing power on the addition of alkali was formed before or just as the solution was becoming reddish, and in view of the fact that definite evidence for the formation of a quinone during the oxidation of adrenaline was lacking, it was thought advisable to see if this substance were of a quinonoid nature and if possible to undertake its isolation and investigate its physiological action.

EXPERIMENTAL.

(1) *The effect of time on the action of alkali on adrenaline.* 2 ml. of a solution of adrenaline 1: 100,000 were treated with 0·1 ml. of N NaOH or 1·5 N Na_2CO_3 for varying times, neutralized, and the reducing power to arsenomolybdic acid determined. The results are shown in Figs. 1 and 2 (Curve 2). The graph shows that the reducing power rose rapidly to a maximum in 30 secs. (90 secs. with 1·5 N Na_2CO_3) and remained constant for about 2 hours and then declined slowly.

(2) *The effect of pH on the reducing power.* 2 ml. of adrenaline (1: 100,000 or 1: 1 million) were treated at different hydrogen ion concentrations for varying times and then the reducing power determined. The results (Fig. 3) show that the maximum reducing power is attained rather quicker with increasing pH but that the maximum is not affected.

(3) *The chemical properties of adrenaline and alkali treated adrenaline (A.T.A.).* A solution of adrenaline 1: 5,000 to 1: 50,000 was treated as in (1) for various times (for the times of the production of the maximum amount of A.T.A. see Fig. 1) and the results of the following tests compared with those of adrenaline alone. As far as can be determined oxygen is necessary for the reaction.

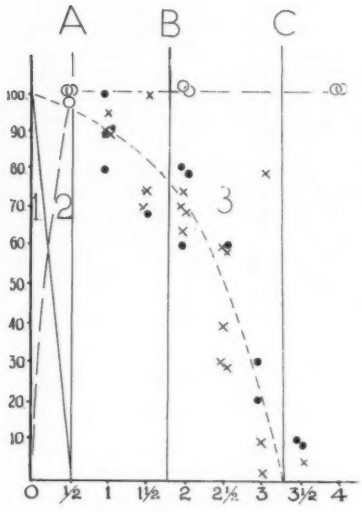


Fig. 1.

Action of N. NaOH on adrenaline.

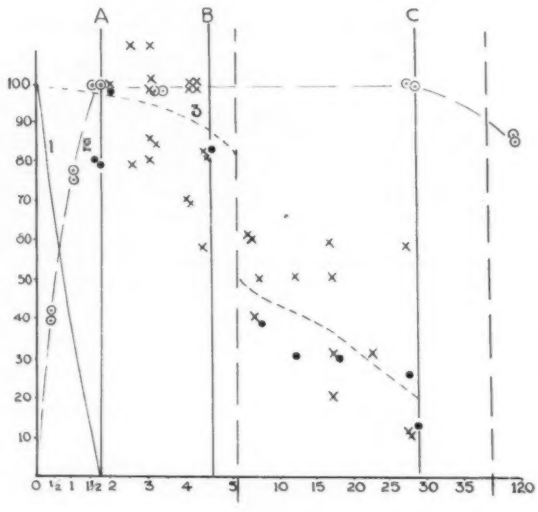


Fig. 2.

Action of 1·5 N Na_2CO_3 on adrenaline.

Curve 1. Hypothetical curve for loss of physiological activity if it were to follow increase in reducing power.

Curve 2. Increase in reducing power due to action of alkali.

Curve 3. Decrease in physiological activity due to action of alkali.

Abscissa—Time in minutes.

Ordinate—Percentage activity, which refers both to increase in reducing power and physiological activity.

• Assay by frog's heart. × Assay by isolated intestine.

The following colour changes were observed, on the addition of NaOH. A pink colour developed and reached a maximum in 30 secs. and then gradually faded to a greenish yellow. With Na_2CO_3 nearly 90 secs. were required for the colour to reach its maximum, it persisted for 2 mins. before gradually changing to a pale yellow.

On the addition of ferric chloride to adrenaline the solution turned very dark green changing to light green and yellow. On the other hand with A.T.A. only a fleeting light green was perceived. The guaiacum test was only slightly positive but there was a large liberation of iodine from acidified potassium iodide. These tests suggest the presence of a quinone grouping. The potassium nitrate and Cole's tests were negative and Pauly's test was positive in both cases. This is notably due to the alkali used in the test. The nitroprusside test was negative in the case of adrenaline but slightly positive in the case of A.T.A. The results of these tests suggest the absence of an indole structure were it not for the positive results obtained with p-dimethylaminobenzaldehyde which gives a pink colour in the presence of indole compounds. This test is apparently more sensitive than the foregoing. With adrenaline the test is negative. The following table shows the results with A.T.A. The relative depths of pink are indicated by crosses.

TABLE 1.

Time after addition of alkali (secs.).	N. NaOH	1·5 N Na_2CO_3.
20	+	—
45	++++	—
60	+++++	—
90	++	+
120	+	++
180	—	+++
300	—	+
600	—	—

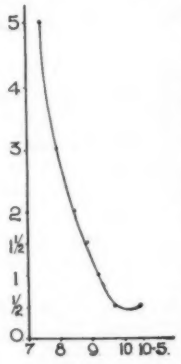


Fig. 3.

Effect of pH on reducing power of adrenaline.

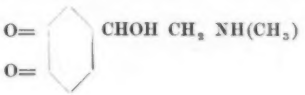
Abscissa—Hydrogen ion concentration.

Ordinate—Time in minutes.

However the depth of colour developed by A.T.A. is only about ⅓th of that developed by an equivalent solution of adrenochrome with p-dimethylaminobenzaldehyde, which suggests that at any one moment only a fraction of the adrenaline has been converted to an indole derivative.

Another observation indicates that the pink colour of an alkaline solution of adrenaline is not entirely due to the presence of adrenochrome. If the leuco base of adrenochrome is treated with potassium iodate and acetic acid the solution takes on a black murky appearance, presumably due to the precipitation of melanin. Under similar conditions a solution of A.T.A. merely becomes very dark red. It should be noted that although the reducing power of neither adrenochrome nor the leuco base is increased by the addition of alkali, their total reducing power is only about 70 p.c. that of an equivalent solution of A.T.A.

The above results lead to the conclusion that A.T.A. is either the simple quinone form of adrenaline or has an indole structure. Because of the evidence given the latter conclusion is unlikely, and in any case A.T.A. possesses considerable physiological activity which is not possessed by adrenochrome. Hence A.T.A. has been given the following tentative formula.



Many attempts by various methods to isolate A.T.A. were unsuccessful, either adrenaline or some physiologically inactive substance being obtained.

Physiological Experiments.

In view of the commonly held opinion that adrenaline is quickly inactivated by oxidation in the presence of alkali and because of the, as yet, unknown chemical nature of Sympathin E and I, the pharmacological activity of A.T.A. was investigated.

To 2 ml. of adrenaline HCl solution (1: 10,000 to 1: 1 million) was added 0·1 ml. N NaOH (pH 9·9) or 0·2 ml. 1·5 N Na_2CO_3 (pH 8·8) and the mixture allowed to stand for various times. It was then quickly neutralized with N HCl (employing phenolphthalein or methyl red dissolved in 1/100 NaOH as internal indicator). Appropriate dilutions were tested on the biological preparations. Controls were run in all cases.

Test objects used were the frog's heart (Straub preparation) and the isolated intestines (rat or rabbit), representing respectively excitatory and inhibitory actions of adrenaline.

The results are given in Figs. 1 and 2 (Curve 3 is a mean of the results obtained with heart and intestine).

It will be seen that during the first 2 mins. action by alkali (4 mins. with Na_2CO_3) on adrenaline, i.e. during the formation of A.T.A., its activity is unchanged; after this time there is a gradual decline in activity but even after 20 mins. there may be as much as 20 p.c. activity remaining. Usually the stronger the adrenaline solution the more resistant it was to alkali.

Investigation was made on the properties of adrenaline and A.T.A. In using their effect on the cat's blood pressure and uterus, either a spinal preparation was employed, or the animal was under Dial anaesthesia.

The results showed that the properties of adrenaline and A.T.A. were identical. Both raised the blood pressure, increased the heart rate and inhibited the uterus. These actions were potentiated by cocaine and antagonized by yohimbine (yohimbine was employed instead of ergotoxine). The effects in the intact animal were even more striking than those with the isolated organs, because in the case of NaOH some activity was still evident after 15 mins. and with Na_2CO_3 after 40 mins.

DISCUSSION.

Evidence is given that during the action of alkali on adrenaline an intermediate is formed which possesses the same physiological properties as adrenaline itself.

Figs. 1 and 2 show that under the influence of alkali the physiological activity of adrenaline does not decrease by more than 10 p.c. for a period of 2 mins. (5 with Na_2CO_3), while within this time a definite change in the reducing power and the emergence of other chemical properties has taken place.

If the increase in reducing power of adrenaline to arsenomolybdic acid were due to the formation of adrenochrome (or some other substance which also was without physiological activity) the position of curve 3 would be that of 1. The fact that curve 1 is displaced to the right means that after the reducing power has attained a maximum (i.e. a definite chemical change has occurred) physiological activity is still retained for several minutes. From this A.T.A. is postulated to exist between times A and B. After time B it is gradually destroyed to a physiologically inactive substance, the destruction being complete at time C. This substance is not adrenochrome as it does not give the p-dimethylaminobenzaldehyde test (see Table 1). Its exact nature is unknown.

The reason why the apparent activity of A.T.A. is only about 90 p.c. that of adrenaline is because, as already explained, a small amount of adrenochrome is formed in a side reaction. The full action may be represented thus:

$$\text{Adrenaline} \begin{cases} \overset{90\%}{\longrightarrow} \text{A.T.A.} \longrightarrow \text{Unknown compound.} \\ \overset{10\%}{\longrightarrow} \text{Adrenochrome} \longrightarrow \text{Leuco base.} \end{cases}$$

Thus the results of physiological investigation confirm those obtained chemically, i.e. there is, at first, only a small change in the molecule and this change must be in the nucleus, because it is well known that any change in the side chain profoundly alters the pharmacological properties of adrenaline (Gunn, 1939). The similarity of the physiological action of adrenaline and A.T.A. can be explained in two ways, either that before adrenaline exhibits its action in the body it is rapidly oxidized to A.T.A. or that A.T.A. is reduced to adrenaline, i.e. adrenaline may undergo reversible oxidation and reduction in the body. This suggestion has been made by various authors (see Welch and Heard, 1935).

It had been hoped that there would have been a qualitative difference in the properties of adrenaline and A.T.A. which could have been connected with Cannon's hypothesis of the dual nature of sympathetic transmission. The results do not offer any help in this direction, but agree with earlier work of Blaschko (1936) that sympathin is probably not an oxidation product of adrenaline, as suggested by Bacq (1935). Furthermore the oxidation of adrenaline to adrenoxine by catechol oxidase (Bacq, 1938) must proceed by a different path because in no case was a substance with such pronounced inhibitory properties encountered, even after prolonged (4 hours) treatment with alkali. In his most recent paper Richter (1939) suggests that *in vivo* adrenaline is destroyed via the nucleus not via alterations in the side chain.

The fact that adrenaline can withstand such a high degree of alkalinity may offer an explanation of certain paradoxes between the "pharmacological" and

metabolic actions of adrenaline. As is well-known an injection of as much as 0·1 mg./kg. will raise the blood pressure and quicken the heart rate for only a few minutes. The older explanation that adrenaline was quickly destroyed has been shown to be incorrect by Bain (1937), who attributes the inactivation to adsorption by the corpuscles. This does not explain why, if rats are injected subcutaneously with 2 mg./kg. of adrenaline, at the end of three hours the liver glycogen will have risen from 6 mg./100 gm. to 42 mg./100 gm. and muscle glycogen to have fallen similarly (Cori and Cori, 1931). However Cori's theory, that adrenaline is not destroyed when injected subcutaneously, but is slowly absorbed over a period which may be as long as four hours, is strengthened by the present evidence that adrenaline can withstand an alkalinity in the region of pH 8·8 for more than half-an-hour, a pH which must be greatly in excess of that in the subcutaneous regions.

SUMMARY.

During the action of alkali on adrenaline a substance is formed which possesses the properties of a quinone but which retains the physiological activity of adrenaline.

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A NOTE ON THE METABOLISM OF CHLOROPLAST PROTEIN

by E. A. HANSON

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(Accepted for publication 25th April, 1941.)

INTRODUCTION.

In investigations of protein metabolism in leaves, the protein has generally been considered as a whole. Yet it is evident that the total protein is probably made up of chemically different units and is in any case distributed among different organs of the cell. Methods, however, have been lacking by means of which plant proteins can be separated into different fractions.

Menke (1938) discovered that by differential centrifugation of plant material chloroplasts may be separated from the rest of the constituents, and this method has been much improved by the subsequent investigations of Granick (1938). We do not yet know whether the chloroplast proteins differ chemically from the other plant proteins, but it may be regarded as possible from the special rôle of the chloroplast in the cell. Hence this "anatomical" separation of protein fractions may lead to a better chemical knowledge of plant proteins; and moreover it is the first step in answering the question where in the cell proteins are synthesized.

The present work represents a preliminary study of the variation of the chloroplast protein in relation to the total leaf protein under different environmental conditions. The material used was the grass *Phalaris tuberosa* L.

METHODS.

Although the purification of chloroplasts by means of fractional centrifugation is a simple method, the procedure has to be adapted according to the particular material used. Fractional centrifugation combined with salt precipitation as carried out by Menke, proved impracticable with *Phalaris*, as addition of any electrolyte disintegrated the chloroplasts in suspension; therefore we had to rely on centrifugation only, and successfully applied Granick's method of grinding and centrifuging the leaf material in 0·5 M saccharose solution. This solution was used also to wash the precipitated chloroplasts and thus free them from all microscopically observable contaminations. By this method described in detail below, it was possible to obtain unbroken chloroplasts to the extent of 4 p.c. of the total number present. The purified chloroplasts remained brightly green for a period of four hours.

Samples of 60 to 80 gm. of fresh leaf material were ground in lots of 15–20 gm. with much coarse sand and about 30–40 ml. 0·5 M saccharose solution, and the green suspension was then decanted. This procedure was repeated twice and the three chloroplast suspensions, amounting to about 100 ml., were combined. Addition of $CaCO_3$ powder to prevent formation of phaeophytin was not necessary, because, as long as the chloroplasts remained unbroken, decomposition of chlorophyll did not take place as shown by the phaeophytin and phase tests.

In the 100 ml. suspension the coarser material was precipitated by 5 minutes' centrifugation at low speed. The supernatant fluid was then centrifuged at 2000*g* for 20 minutes or less, the fluid discarded, and the green top of the precipitate dissolved in 10 ml. of fresh 0·5 M saccharose solution. This now much purer suspension was transferred to a centrifuge tube of test-tube width with a tube, 5 mm. wide and 5 cm. long, sealed on to the bottom. After centrifuging for 20 minutes or longer at 2000*g* the different layers were easily collected from the narrow bottom tube by means of a capillary pipette and tested microscopically. The best layer determined by microscopic examination was collected in a similar centrifuge tube but with a bottom tube of only 3·5 mm. width. After centrifuging for 20 minutes or longer the best layer was again collected. It no longer contained contaminants if the procedure was carried out well. More centrifugations could not be performed because after four hours the chloroplasts suddenly assume a dull appearance and disintegrate. The process of disintegration takes about 30 minutes to complete.

From this chloroplast material the pigments were extracted by means of 90 p.c. or stronger acetone. The chlorophyll was estimated colorimetrically as ethyl chlorophyllide with a preparation of the pure substance as a standard. The residue of the acetone extraction was washed with tungstic acid and the nitrogen estimated by means of the micro-Kjeldahl method.

Other aliquots of the fresh material were used for estimating dry weight and also total leaf protein by the method described by Petrie and Wood (1938).

EXPERIMENT A.

Seeds were sown in a nitrogen-deficient sand culture, in small glass pots on May 31, 1939. On September 9 (day 0), 25 days before the exsertion of inflorescences, certain pots were given an additional ammonium sulphate dose. The results are given in the accompanying table.

TABLE 1.

1	2	3	4	5	6	7	8	9	10	11
Harvest.	Treatment.	Time in days.	Total protein in mg. per 5 gm. fresh material.	Total protein in mg. per pot.	Plastid protein in mg. per 5 gm. fresh material.	Plastid protein p.c. of total protein.	Chlorophyll in mg. per 5 gm. fresh material.	Plastid protein / Chlorophyll.	Dry wt. in gm. per 5 gm. fresh material.	Appearance of plants.
Experiment A.										
1	Untr.	0	111	401	40·0	36	8·0	5·0	0·880	Good, rather pale.
2	+ N	7	180	748	62·0	34	11·7	5·3	0·895	Very good.
3	Untr.	7	135	597	44·5	33	9·3	4·8	0·895	Good.
4	+ N	16	205	—	65·3	32	12·2	5·4	0·820	Very good.
5	+ N	21	213	—	67·0	31	12·4	5·4	0·860	Very good.
6	Untr.	25	101	461	35·8	36	7·9	4·5	1·005	Slightly worse than at harvest 1.
Experiment B.										
1.	Untr.	0	206	1,355	50·5	24	9·8	5·2	0·995	Good.
2	— H_2O	4	210	1,428	51·7	25	9·8	5·3	1·020	Good, no wilting.
3	— H_2O	9	231	1,284	39·3	17	9·1	4·3	1·150	Wilting, yellowing.
4	— H_2O	11	217	1,084	27·0	12	6·0	4·5	1·200	Badly wilting, yellowing.
5	Untr.	14	215	1,574	—	—	11·0	—	1·075	Good.

The amounts of protein are expressed in mg. assuming a nitrogen content of 16%.

Results. At the beginning of the experiment the absolute protein content of the leaves was still increasing (column 5). Ammonium sulphate treatment accelerated this increase in both chloroplasts and the rest of the cell, the extra-chloroplast protein being accelerated relatively more (column 7): in the untreated pots the ratio of the two proteins remained constant. The protein-chlorophyll ratio of the plastids (column 9) fell slightly during the experiment in the untreated plants: treatment resulted in an increase in the ratio. At the final harvest, when this ratio had fallen to 4·5, the plants could no longer be called normal: they were yellowish and stunted, whereas plants with a ratio of more than 5·0 were deep green and succulent.

Discussion. This experiment is in agreement with data given by Chibnall (1939) who recovered 40 p.c. of the leaf protein in *Spinacia* from the chloroplasts. In healthy *Phalaris* plants the recovery proved to be only slightly less than this. The experiment shows, however, a slight disagreement with data given by Smith (1940), who found a constant relation in his purified chloroplast material of about 16 parts of chlorophyll to 100 parts of chloroplast protein (ratio 6·25). Smith worked with *Spinacia* and *Aspidistra*, plants which might possibly show higher ratios than *Phalaris*; but he used salt-precipitation and centrifugation as purification methods and it was found in the present experiment that addition of any salts disintegrated *Phalaris* chloroplasts rapidly, thus rendering purification impossible. However, although neither methods nor material are directly comparable, the results do not disagree very greatly. On the other hand in *Phalaris* the ratio is certainly affected by culture conditions as described above.

The ratio in *Phalaris* varied between 4·5 and 5·4. In general in enzymes a stoichiometric relationship exists between the prosthetic group and the protein; and, in the case of the tetrapyrrol compounds to which class chlorophyll belongs, four tetrapyrrol groups are always found to be attached to a protein carrier of molecular weight 68,000 (Hanson, 1938). It may be expected

that a similar relationship will hold for the synthetic mechanism in the chloroplast, although the molecular weight may be different. In the present data the protein-chlorophyll ratio of the plastids is variable, but we can nevertheless calculate a maximum value for the relationship. It is known that the molecular weight of chlorophyll is 915; therefore, assuming that the molecular weight of the protein carrier is 68,000 as in animal life, and that its nitrogen content is 16 p.c., it can be seen that the maximum number of chlorophyll molecules permitted by the data per protein molecule is 16. This evidently leaves a certain excess protein which is presumably uncombined with chlorophyll. The amount of this protein is too large to be due to any non-protein nitrogen which might have been present in the chloroplasts and precipitated by tungstic acid.

It may therefore be concluded that the plastid contains a protein surplus which increases with ammonium sulphate treatment, that is, when net protein synthesis is rapid, and which may be available for breakdown and transfer to the rest of the protoplasm. As there is a tendency to regard plastids and nuclei as centres of production in the cell it is perhaps reasonable (with due reserve because there are so far no proofs, only many indications) also to look upon the chloroplast as one of the protein producers of the cell.

EXPERIMENT B.

In this experiment seeds of *Phalaris* were also sown on May 31 in a medium containing adequate nitrogen, and plants were first harvested on October 9 (day 0). Certain pots were submitted to reduced water supply, u[nder w]hich conditions it was expected from the work of Petrie and Wood (1938) that net protein [breakdown] would occur in the leaves. Thus nitrogen supply and ontogenetic stage, as well as the [rate] of the change in protein content brought about experimentally, were different in this ex[periment] from the previous one.

Results. In Experiment B also the pro[tein-chl]orophyll ratio in the chloroplasts was far from constant (column 9). In normal plants (har[vest 1] and in plants till then little affected by lack of water (harvest 2) a ratio of intermediate m[agni]tude, 5·2–5·3, was maintained. In wilting plants, however, the ratio became extremely low. The percentage of chloroplast protein also fell (column 7); as there was a simultaneous fall of total leaf protein (column 5) a net hydrolysis of leaf protein must have taken place, and it is noteworthy that this hydrolysis was faster in the plastids than in the rest of the cell.

This experiment was planned to be a preliminary one, but it suggests that, under conditions leading to net protein loss from the leaf, the plastids are more liable to disorganization than the rest of the cell, and the protein carrier evidently undergoes hydrolysis.

SUMMARY.

Chloroplasts of *Phalaris tuberosa* were prepared by a centrifugation method which is described. It was found that the protein of the chloroplasts represented approximately 36 p.c. of the total leaf protein. The protein-chlorophyll ratio of the chloroplasts themselves increased during net protein synthesis and decreased during net protein hydrolysis. If the photosynthetic mechanism involves a constant ratio of protein to chlorophyll in the plastids, the data suggest that a certain surplus protein is produced in the chloroplast when the nitrogen supply is abundant. Under conditions of drought the chloroplasts lose their protein more rapidly than the rest of the cell.

Acknowledgments. The writer desires to express his indebtedness to Professor J. A. Prescott for permission to carry out this work at the Waite Institute, and to Dr. A. H. K. Petrie for critical suggestions during the performance of the work and preparation of the manuscript.

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AN AEROBIC, SPORULATING BACILLUS THAT FORMS ROTATING AND MIGRATING COLONIES

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(Accepted for publication 15th April, 1941.)

INTRODUCTION.

Migration of bacterial colonies over the surface of agar media was first described by Muto (1904) when studying a non-sporulating bacterium isolated from saliva. This observation apparently attracted no attention. The phenomenon was rediscovered by Roberts (1935) when studying a terminal-spored bacillus to which he gave the name *B. rotans*. Colonial migration has also been described for other terminal-spored aerobic bacilli (*B. circulans, B. alvei*, and some undetermined bacilli) by Smith and Clark (1938), Russ-Münzer[1] (1938) and Shinn (1938).

In this paper a similar bacillus isolated in Australia is described. It is related more closely to *B. circulans* than to *B. rotans, B. alvei* or *B. helixoides*,[2] but is probably not identical with it.

DESCRIPTION OF THE BACILLUS.

It was first isolated by us in Melbourne in 1937 from a contaminated culture of an anaerobe in Hall's cooked brain medium and is referred to as strain A. Eighteen months later another closely related strain, referred to as strain B, appeared on an uninoculated blood agar plate incubated overnight.

The characteristics of the bacillus are as follows:

Isolation. Laboratory contaminant.

Morphology. On blood agar, after 5 hours at 37° C., it forms short rods, single or in pairs, straight, from 3 to 4μ long and from 0·25 to 0·3μ wide, with rounded or slightly tapered ends. Tapering increases with age. The rods tend to lie together in groups (Fig. 1, No. 1). On Loeffler's serum some filaments up to about 500μ in length are formed. It is motile, and has numerous fine peritrichial flagella which are easily torn off (Fig. 1, No. 2). After a few days on plain agar or Loeffler's serum it forms large (1·7μ by 0·4μ) oval subterminal or terminal endospores. These are usually found free and stain readily with cold dilute carbol fuchsin. Capsules are not formed. Young forms stain irregularly in a barred manner and are weakly Gram-positive. They quickly become Gram-negative.

Motility. There is no evidence of co-ordinated movement in fluid cultures. Many varieties of progression occur simultaneously in the one hanging-drop microculture preparation: long filamentous forms tend to move in a slow serpentine manner; short forms may rotate around a longitudinal or a transverse axis, oscillate from side to side, or tumble irregularly.

Cultural Reactions.

Agar stroke. When complete, growth is butyrous, confluent, slightly spreading (mostly due to colonial migration), raised, with a bevelled margin, abundant and with a glistening lustre. Fully developed colonies (Fig. 1, No. 11) on agar are circular with a radially streaked or a contoured surface, are up to 2 mm. in diameter, and have an amorphous structure. The edge is lobate to various degrees. They are transparent and appear light honey-brown by transmitted light and whitish by reflected light. Colonies are easily emulsifiable and do not colour the medium.

Gelatin stab. Growth is abundant, filiform, opaque, with a wide zone of delicate cloudy outgrowths for some millimeters from the track. Growth extends to the full depth of the track, with further penetration into the surrounding medium at the top and on the surface. There is no liquefaction, even after three weeks at 37° C.

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[1] According to Clark (1939), Russ-Münzer's bacilli have been identified by Smith as *B. circulans* and *B. alvei*.

[2] So named by Muto; strict latinity demands "helicoides".

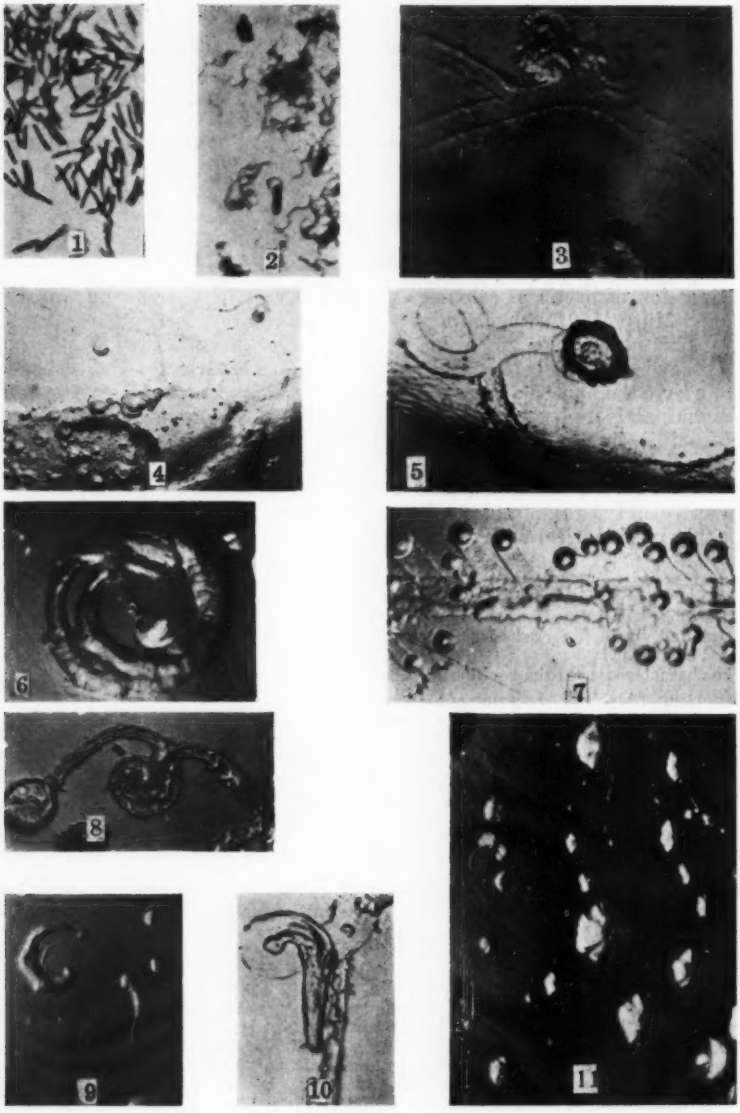

Fig. 1.

Agar shake culture. In 1·5 p.c. Lemco- or V.F.-agar the colonies grow throughout the medium, but are larger in the zone within one centimetre of the top. Colonies are lenticular in V.F.-agar, irregular in Lemco agar.

Sheep-blood (2 p.c.) agar plates. There is good growth but no haemolysis or other change. *McConkey plates.* The colonies are pink. Growth is better at room temperature than at 37° C. *Meat infusion broth.* Marked turbidity occurs throughout the medium, with a granular and viscous deposit in six days. *Serum broth.* The medium becomes markedly turbid, and a granular deposit forms. A slight pellicle appears later. *Liver piece broth.* The medium becomes turbid; no gas develops. *Hall's cooked brain medium.* There is no gas or blackening. *Ditto plus iron.* Blackening occurs after six days. *Litmus milk.* The litmus is reduced in 24 hours, but there is no other obvious change in the medium. *Zeissler's milk.* No change is observable. *Rupprecht and Rottgard's liver piece milk.* Slight deposition of floccules occurs around the liver particles after three days at 37° C. *Loeffler's serum medium.* Growth is moderate, but no pigment is formed and no digestion or softening occurs. *Potato.* There is good growth, but no pigment is formed.

Biochemical reactions. Catalase is produced. The methyl-red test and the Voges-Proskauer test are negative. Nitrates are reduced to nitrites. Indol is not produced. Hydrogen sulphide cannot be demonstrated in cultures in peptone water but, from the delayed blackening of cooked brain medium with iron, appears to be formed in small quantities.

Fermentation reactions. In broth with Andrade's indicator plus 1 p.c. of test substance this bacillus produces acid, but not gas, from arabinose, xylose, glucose, levulose, mannose, galactose, saccharose, maltose, lactose, raffinose, starch, glycogen, glycerol, mannitol, salicin, amygdalin. No change is produced with rhamnose, erythritol, adonitol, dulcitol, sorbitol and inositol. To compare these reactions with those of *B. rotans* as reported by Roberts, who used only 0·5 p.c. of test substance, our bacillus was tested in Andrade's broth with 0·5 p.c. of glucose, saccharose, lactose, maltose and salicin; here again definite acid reactions were obtained.

Strain B differed from strain A in not fermenting glycerin and giving a much slower reaction with raffinose.

Pathogenicity. The intramuscular injection of 2 ml. of a 24-hour culture in broth had no effect on guinea-pigs.

ROTATIONAL AND MIGRATIONAL PHENOMENA.

Two sorts of co-ordinated motility are displayed by this organism: rotation and migration.

Rotation may occur very early during growth, even after four or five hours at 37° C. The cells segregate in small groups and align themselves concentrically around a common centre to form disc-like plaques one cell or a few cells thick. The rate of rotation is greater in smaller groups and the direction of rotation may be either clockwise or anti-clockwise. As multiplication proceeds successive layers are gradually built up in terrace fashion and the colony grows in height; the edges of the "terraces" become indistinguishable and the rotating colony becomes circular, entire, convex and up to 0·5 mm. in diameter.

These colonies may then begin to migrate. Usually they cease rotating while migrating.

LEGEND TO FIGURE 1.

No. 1. Smear, 24-hr. agar culture, Gram stain. × 1360.

No. 2. Smear, 23-hr. agar culture, Levenson's method, showing flagella. × 1360.

No. 3. 24-hr. culture on blood agar showing intersecting tracks somewhat grown up and colonies that have developed after ceasing to migrate. × 10.

No. 4. 48-hr. culture on blood agar, showing two intersecting tracks. The colony at the right has ceased migrating. × 3.

No. 5. 24-hr. culture on blood agar showing track, at the end of which is a developed colony wider than the track. × 10.

No. 6. 24-hr. culture on blood agar showing effect produced when a large colony migrates along a circular or spiral path of small radius. × 10.

No. 7. 72-hr. culture in McIntosh and Fildes' Jar, showing migration of rotating colonies along short straight paths. × 10.

No. 8. 48-hr. culture on blood agar, showing the up-grown track of a wandering colony that has apparently rested temporarily after a slightly curved path, and later begun a spiral path, eventually coming to rest and increasing greatly in size. × 10.

No. 9. 23-hr. culture on blood agar showing a migrating colony. × 10.

No. 10. 48-hr. culture on blood agar showing a migrating tongue-shaped colony. × 10.

No. 11. 24-hr. culture on blood agar showing a migrating colony moving through the swarm film. × 10.

However, particularly when allowed to develop at room temperature or when grown under anaerobic conditions, they may continue to rotate slowly while migrating. Presumably the lower strata of cells are responsible for the translatory movement and carry the slowly rotating upper strata with them. Such colonies usually travel only short distances and in straight lines. (Fig. 1, No. 7).

Migration of groups of cells, from some hundreds to many thousands, is also common. These leave the main growth without going through a phase of rotation, and align themselves parallel to the direction of migration where they form elongated, tongue-shaped masses or microcolonies (Fig. 1, Nos. 4, 9 and 10). These are usually from 0·2 to 0·3 mm. in width, are narrow and rounded in front and end irregularly behind. This type of colony migrates very actively.

When a colony migrates it leaves a peculiar "track" recalling that left by a minute snail. A small number of cells are left behind, mostly at the edges of the track, forming two parallel lines separated by the width of the moving colony. On further incubation many of these discarded cells multiply and the track becomes more obvious (Fig. 1, Nos. 3 and 8).

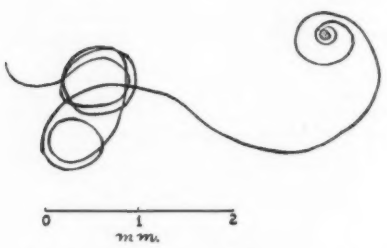

Fig. 2. Sketch of convoluted track of a wandering colony, showing two series of clockwise spirals followed by a final anti-clockwise spiral. The colony had increased considerably in size after coming to rest and showed curved radial markings indicating rotation. The total length of the track was about 2 cm.

Typical migrating colonies, especially the small active type, pursue curved or spiral paths which are often very elaborate and of relatively great length, even two or three centimetres (Fig. 2). Migration may be in a clockwise or anti-clockwise direction. The direction may change several times. After wandering for a variable distance a colony may approach the centre of its spiral path with rapidly shortening radius, cease to migrate, begin to rotate around its centre, lose its elongated shape and increase in size to several times the width of the track at the end of which it is found. On the other hand, colonies may merely cease migrating when pursuing a more or less straight path and then increase in size (Fig. 1, Nos. 5 and 8).

When colonies continue to rotate slowly while migrating the tracks left behind are characteristically marked by close transverse ares of discarded cells.

True swarming rarely occurs on 2 p.c. agar, unless it is moist, and is then only slight. It may occur to a limited extent between the colonies on a crowded plate, when it is not unusual to find that centres of migration develop in the irregularly confluent or fenestrated film-like growth. Groups of cells flock together and push their way through the film, leaving a clean, almost bacteria-free path with raised edges behind them (Fig. 1, No. 11). Migrating colonies may also move out beyond the swarm film and invade the clear medium (Fig. 1, No. 4).

Colonial rotation and migration may occur at all temperatures between 37° C. and 18° C., and under anaerobic as well as aerobic conditions. Not all colonies are motile, but we have not been able to obtain variants that constantly give rise to non-motile colonies. Migration occurs in the presence of 0·1 p.c. sodium luminal but is completely inhibited by 0·25 p.c.

The rate of migration is very much greater in small than in large colonies. It has reached 2·5 mm. per minute in a plate incubated at 37° C. and examined immediately after removal from the incubator. The speed decreases with fall of temperature but is still observable at room temperature.

Impression preparations made according to the technique described by Bissett (1938) show that the motile colonies consist of rods not markedly different from those in non-motile colonies. Young, tongue-like, wandering colonies usually have some relatively long forms especially at the sides and the anterior end, but the majority of the rods are normal, short forms.

On 1 p.c. agar the growth is entirely different. The bacillus swarms at temperatures from 22° C. to 37° C., more rapidly at the higher temperature. It shows no disposition to form separate colonies nor to migrate as above. The growth is film-like and not as abundant as on 2 p.c. agar. On continued incubation as the medium dries out, some attempt at colony formation may occur at points in the film-like swarm growth.

Ability to migrate on concentrated agar plates. An interesting comparison was made between the relative abilities of our bacillus to migrate over agar plates with increasing agar concentration and of *Pr. vulgaris* to swarm over similar plates. A series of nutrient agar plates was prepared with concentrations of agar rising by 0·5 p.c. from 1·5 p.c. to 5 p.c. Plates of each concentration were sown in the centre with a loopful of a culture of our bacillus and of *Pr. vulgaris* respectively and incubated at 37° C. The results after 24 hours were as follows:

Agar p.c.	Our bacillus.	*Pr. vulgaris.*
1·5	Good migration, with swarming between colonies.	Plate covered; 2 swarm waves to cover plate.
2·0	Good migration; no swarming.	Plate covered; 4½ waves to cover.
2·5	" " " "	Plate covered; 6 waves to cover.
3·0	Moderate degree of migration.	Only half of plate covered; 7 waves.
3·5	" " " "	Zone of swarming only 1 cm. wide; 7 waves.
4·0	" " " "	Very narrow zone of swarming 3 mm. wide; difficult to distinguish waves.
4·5	" " " "	No swarming.
5·0	" " " "	" "

Under our conditions of cultivation, in no case does colonial migration cause complete covering of the plate; often it is of only limited extent. However, it is significant that although swarming of *Pr. vulgaris* was almost inhibited by 3·5 p.c. agar and totally inhibited by agar concentrations beyond 3·5 p.c., our bacillus was still able to produce motile colonies on 5 p.c. agar.

DISCUSSION.

In comparing this bacillus with *B. rotans*, the main differences, in so far as they can be judged from Roberts' description, are as follows:

B. rotans Roberts.	Our bacillus.
No acid with mannitol or glucose.	Acid with mannitol and glucose.
Alkaline reaction in all carbohydrate media tested.	Acid reaction in all carbohydrate media which are attacked; alkaline reaction in none.
Nitrates not reduced.	Nitrates reduced.
Litmus milk slightly reduced after 10 days.	Litmus milk reduced in 24 hours.
Gelatin completely liquefied in 30 days.	Gelatin not liquefied.
Growth poor at 37° C.; colonies non-motile.	Grows well at 37° C.; colonies strongly motile.

The alkali-producing mildly proteolytic characteristics of *B. rotans* differentiate it from the acid-producing saccharolytic non-proteolytic characteristics of our bacillus, as does the rapid liquefaction of gelatin by Russ-Münzer's bacillus, of which no detailed description has yet appeared. *B. circulans*, according to Bergey, Breed, Murray and Parker Hitchens (1939),[3] decolorizes slowly, acidifies and clots litmus milk: our organism does not acidify and clot milk. There are also numerous points of difference between it and *B. alvei*. The scanty description given by Muto (1904) of *B. helixoides* makes it difficult to compare it with our bacillus, but there is little doubt that they are quite distinct.

It seems that our bacillus is more closely related to *B. circulans* than to *B. rotans*, but further investigation is necessary to determine its relationship more exactly.

We have been unable to carry out cinematographic observations on moving colonies, as Shinn (1938) did with those of *B. alvei*; therefore we are unable to add much to the description of the mechanism of colonial rotation and migration. Muto claimed that with *B. helixoides* one bacillus in each colony acted, in his words, as "Führer" of the group. Impression preparations of our bacillus have not given evidence that this occurs with our organism. It seems probable that the colony is held together by some intercellular fluid in which the stimulus for co-ordinated flagellar movement is transmitted. When the proportion of agar is less than 1·5 p.c., the cohesive and co-ordinating mechanism is not sufficiently powerful, and uncontrolled swarming occurs. It is also probable that only the layers of bacilli close to the agar surface have the necessary alignment in the direction of colonial movement, and that this moving layer carries the remainder of the colony, which may even be rotating.

Colonial migration probably gives increased opportunities for dissemination under difficult conditions. Lehmann and Neumann (1927) have stated that "from

[3] Jordan's original type strain does not appear to be available to-day. He reported liquefaction of gelatine, but apparently carried out no fermentation tests; Ford (1927), from a bacillus which he considered "corresponded closely with Jordan's description", states that it does not liquefy gelatine; in addition he gives some fermentation reactions. Neither author described motile colonies, but merely rotatory movement of the rods in the colonies.

a practical point of view movement of bacteria due to flagellar motion is, because of its absolute slowness (the fastest movement is 1 mm. in 20 seconds), of no great importance, particularly since it is not directed (or co-ordinated)". This statement, particularly its last part, may have to be modified in the light of our present knowledge. All motile organisms are able to spread over the surface of "solid" media by active centripetal movement, if the viscosity is reduced or the amount of free surface moisture is increased, as by adding gelatin to the agar (Dimitrijevic-Speth, 1929; Ivanic and Dimitrijevic-Speth, 1931) or reducing the proportion of agar in media (Gard, 1938). Even when such special conditions do not prevail, as on ordinary 2 p.c. agar media, active swarmers like *Proteus vulgaris*, by virtue of their high motility and primitive but effective group motility, are still able to outstrip motile competitors that can then grow only as localized colonies where chance has placed them.

Occupying an intermediate position between these are organisms such as that we have described, which have only a poorly developed capacity for swarming but have developed to a high degree the capacity to form motile colonies. This is demonstrably more effective than swarming as a spreading mechanism under conditions of relative desiccation, as when high concentrations of agar are used.

It seems that each method of spread has its advantages: under conditions of moderate moistness, "swarmers" like *Pr. vulgaris* have the advantage over the "wanderers", such as our bacillus; when the conditions become drier, the "swarmers" are immobilized but the "wanderers" are still able to move bodily as entire colonies over the nutrient surface.

SUMMARY.

A bacillus that produces motile colonies on agar media is described. It differs from *B. rotans* Roberts, 1935, *B. alvei* and *B. helixoides* Muto, 1904, but is related to *B. circulans* Jordan, 1890.

The biological significance of colonial motility is discussed.

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